Doctoral Thesis

Molecular dissection of the yeast replication fork barrier II: in vitro reconstitution of the replication fork barrier

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Molecular Dissection of the Yeast Replication Fork Barrier:
I: Architecture of the Stalled Replication Fork
II: In vitro Reconstitution of the Replication Fork Barrier

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The ribosomal RNA (rRNA) genes of *Saccharomyces cerevisiae* are organised in a single cluster of about 150 tandemly repeated transcription units. During the S-phase, DNA replication initiates in the nontranscribed sequences that separate two adjacent transcription units. Replication forks moving opposite to the direction of the transcription are arrested at the 3' end of the transcription units. This replication fork barrier (RFB) constitutes the first step of replication termination, because a replication fork from the adjacent replicon merges with the stalled fork at the RFB. So far, the RFB is the only well-defined replication termination site in the linear chromosomes of yeast and higher eukaryotes. This makes the RFB an ideal target to study eukaryotic replication arrest. Furthermore, replication arrest at the RFB seems to be functionally linked to the processes of transcription and recombination, which overlap temporally and/or spatially. Two distinct approaches to the topic were applied. In one we sought to characterise the structure and position of the stalled replication fork at nucleotide resolution. Another approach aimed at identifying molecular components that mediate the replication arrest at the RFB, in order to elucidate the molecular mechanisms of replication arrest as well as functional links to transcriptional and recombination processes.

By means of preparative two-dimensional (2D) gel electrophoresis, we isolated replicative intermediates (RIs) that were highly enriched for replication forks stalled at the RFB. The positions of the nascent strands at the RFB were determined at nucleotide resolution. We found a major arrest site overlapping the *HpaI* restriction site at the border of a *HpaI*-HindIII fragment essential for the RFB, and two minor, closely spaced arrest sites within this fragment. Strikingly, the nascent lagging strand is extended three bases farther than the nascent leading strand. Furthermore, the majority of the stalled lagging strand was found to be completely processed. Thus, the stalled RIs at the RFB hardly expose any single-stranded DNA on its newly synthesised arms. A model explaining the relative positions of the nascent strands is presented. Finally, we have digested the RIs with T4 endonuclease VII, an enzyme recognising branched DNA molecules. This enzyme cleaves in the newly synthesised arms, thereby specifically releasing...
the leading arm. Interestingly, no digestion products indicative of Holliday-like intermediates, a DNA structure recently proposed to occur at the RFB, were detected. This suggests that only very few, if any, of the purified RIs adopt such a structure. Thus, in combination with 2D gel electrophoresis and high-resolution analysis of the nascent strands, T4 endonuclease VII provides a useful tool to analyse replication forks.

In order to set up an in vitro system, in which the RFB can be reproduced, sequences from the 3’ end of the yeast rRNA genes that are required for a functional RFB, were fused to an SV40-based vector. The constructs were replicated in vitro in the presence of SV40 T-antigen and a cytosolic extract prepared from human cells. The progression of the replication forks was monitored by 2D gel electrophoresis. No pausing of replication forks at the introduced yeast sequences was observed, suggesting that the yeast sequences as such do not impede the replication fork progression, and that no factors capable of complementing a missing yeast factor are present in the human cytosol. Addition of a crude whole-cell yeast extract completely abolished in vitro replication. The extract was fractionated by chromatography and the individual fractions were monitored for proteins binding to the DNA sequence required for the RFB by electro-mobility shift assays (EMSAs). A DNA binding activity specifically binding to this sequence was identified and partially purified. No other proteins had been reported to bind that sequence so far. In parallel, a protein termed Fob1p, which had been shown to be required for the RFB, was cloned and overexpressed in E. coli. Using EMSAs, a weak, but specific binding of the DNA sequence required for the RFB could be assigned to the recombinant Fob1p. Furthermore, in vitro replication of a plasmid with four consecutive RFB sequences in the presence of purified Fob1p, revealed a slowing down of the replication fork at the repeated sequences. Thus the Fob1p might be a DNA binding protein that, possibly with more protein(s), mediates the efficient replication fork arrest that was observed in vivo. Finally, a polyclonal α-Fob1 antibody was raised in rabbits. The antibody has been tested and characterised. Immunoprecipitation experiments showed that only tiny amounts of Fob1p are present in the crude whole-cell yeast extract. In combination with SDS-PAGE analyses, this suggests that the DNA binding activity identified in fractionated yeast extracts and Fob1p are probably not identical.
ZUSAMMENFASSUNG


Zusammenfassung


DNA vom 3' Ende Hefe rRNA Genen wurden in einem auf dem SV40 Virus basierenden Vektor eingefügt, um mit Hilfe dieser Konstrukte die RFB in einem in vitro System nachzubilden. Diese Konstrukte wurden mit Hilfe von T-Antigen und einem zytosolischen Extrakt, der aus Zellkulturen menschlicher Herkunft hergestellt wurde, repliziert. Die Wanderung der Replikationsgabel wurde mittels 2D Gelelektrophorese verfolgt. In diesem System konnte keine RFB beobachtet werden, was darauf schliessen lässt, dass die DNA Sequenzen allein eine Replikationsgabel nicht aufhalten können, sowie dass keine menschlichen zytosolischen Faktoren allfällige Hefefaktoren komplementieren können. Als allerdings ein Hefe-Rohextrakt zur Reaktion gegeben wurde um Hefefaktoren zu bereitzustellen, konnte keine in vitro Replikation mehr festgestellt werden. Der Extrakt wurde darauf mittels Säulenchromatographie fraktioniert und die einzelnen Fraktionen mittels „electromobility shift assays“ (EMSAs) auf Proteine hin untersucht, die an die RFB-DNA-Sequenz binden. Dabei konnte eine Aktivität, die spezifisch an diese Sequenz bindet, identifiziert und partiell gereinigt werden. Bisher sind noch keine Proteine bekannt, die an diese Sequenz binden. Parallel dazu wurde ein für die RFB essentliches Protein, Foblp, in E. coli überexprimiert und gereinigt. Mittels EMSAs konnte gezeigt werden, dass dieses rekombinante Protein zwar schwach, aber spezifisch an die RFB-DNA-Sequenz bindet. Ausserdem wurde eine Verlangsamung der Replikationsgabel in vitro beobachtet, als ein Vektor mit vier konsekutiven RFB-
Zusammenfassung

DNA-Sequenzen repliziert wurde. Das lässt darauf schliessen, dass Fob1p ein DNA bindendes Protein ist, das, womöglich zusammen mit anderen Proteinen, für die RFB verantwortlich ist. Schliesslich wurde ein aus immunisierten Kaninchen gewonnener, polyklonaler α-Fob1 Antikörper getestet und charakterisiert. Nur sehr geringe Mengen an Fob1p konnte durch Immunopräzipitations Experimente im Hefe-Rohextrakt nachgewiesen werden. Dies und SDS-PAGE Analysen deuten darauf hin, dass die DNA bindende Aktivität, die in partiell fraktioniertem Hefeextrakt gefunden wurde, nicht mit Fob1p identisch ist.
1 GENERAL INTRODUCTION

DNA replication is a complex process that ensures the faithful duplication of a cell's entire genome and, in its function, it is central to all cellular organisms. The replication process can be divided into three distinct steps: initiation, elongation and termination. Whereas extensive research has brought about considerable advances in the elucidation of the molecular mechanisms involved in the first of these steps, replication initiation, much less is known about the last, conclusive step of DNA replication. This step, replication termination, can be defined as the sequence of events that occur when two converging replication forks meet and conclude a cycle of DNA replication (Hill, 1992). In the literature, the word "termination" is often used interchangeably with the phrase "replication arrest", even though, in the strictest sense, this is only the first step of replication termination. The present work deals with replication arrest, and thus, the term "replication termination" refers here mainly to the first step of replication termination, to replication arrest.

In the course of my studies I have investigated the structure and molecular mechanism of a site-specific replication fork arrest in the rRNA genes of Saccharomyces Cerevisiae. This replication fork barrier (RFB), as it is commonly referred to, constitutes the first step of replication termination that occurs within the rRNA gene cluster of Saccharomyces Cerevisiae and, most likely, of almost all eukaryotic organisms. To our knowledge, the RFB is the only known site where replication termination occurs within a short, defined DNA sequence in a eukaryotic chromosome. This makes the RFB a site of special interest, offering the unique opportunity to study eukaryotic replication termination at molecular level in the chromosomal context of eukaryotic organisms.

I have divided this work into two separate parts, each containing a summary and an introduction of its own. In the first part I have analysed the stalled replication fork at nucleotide resolution, and in the second part, I have dissected the molecular mechanism of the RFB. The two parts correspond to two distinct projects, and it was my intention that the reader should be able to read each part separately. The purpose of the general introduction is to give an overview of replication termination by summarising the re-
ported data about it, with a special focus on the early step, that is, replication fork arrest. The first chapter deals with replication termination in bacteria, because it is in *B. subtilis* and *E. coli*, where this process is most extensively studied and probably best understood. The second chapter deals with replication termination in eukaryotic organisms, in which much less is known at present. A more specific introduction dealing with the individual projects is presented in the respective chapters.

### 1.1 Replication Termination in Prokaryotic Cells

In bacteria, termination of replication usually involves a replication fork arrest at specific DNA sequences, the replication termini or *Ter*-sites (for reviews see (Baker, 1995; Bastia and Mohanty, 1996; Bussiere and Bastia, 1999; Hill, 1992)). The best-studied examples for sequence specific replication arrest are those of *B. subtilis* and *E. coli*. The first replication termini to be discovered were those of the *E. coli* plasmid R6K (Crosa et al., 1976; Lovett et al., 1975). Subsequently, similar sites were found in the chromosomes of *E. coli* and *S. subtilis* (Kuempel et al., 1977; Louarn et al., 1977; Smith and Wake, 1992; Weiss and Wake, 1984; Weiss and Wake, 1984). In both organisms chromosome replication initiates at a single origin, from where two replication forks diverge until, at about 180° from the origin, they converge in a chromosome region that is referred to as the terminus. Multiple *Ter*-sites are dispersed over the terminus region of the chromosomes of *E. coli* and *B. subtilis*. They are organised as two opposed groups around the central portion of the terminus region (Kuempel et al., 1989; Franks et al., 1995). An approaching replication fork travels unimpeded through the first, origin proximal set of *Ter*-sites. However, if it reaches the second, inversely orientated set of *Ter*-sites the replication fork is restrained. Thus, the polarity and the arrangement of the *Ter*-sites provides a “replication fork trap”, which ensures that a fork can enter the terminus region but not exit it, i.e. the two forks always meet and fuse within the terminus region.

The function of the *Ter*-sites is entirely dependent on the binding of a trans-acting factor. In the case of *E. coli*, the Tus protein (for terminus utilisation substance)
(Hill et al., 1989; Sista et al., 1989) recognises the 22 bp Ter-site, whereas the analogous 47 bp Ter-site of B. subtilis are bound by a protein called RTP (replication termination protein) (Lewis et al., 1990). Interestingly, neither the Ter-sites of E. coli and B. subtilis nor their corresponding DNA binding proteins share any homology. It seems that the two termination systems have evolved independently to achieve the same end. Another striking difference was found in the mode the two proteins bind their cognate sequences. The Tus-protein binds its cognate site as a monomer, whereas RTP as homodimer.

A considerable amount of information on the mechanism of site-specific replication termination in bacteria has been gathered through in vitro experiments using purified terminator proteins (Hill and Marians, 1990). Although no in vitro replication system is currently available for the B. subtilis, the in vitro system of E. coli largely compensated that deficiency, since RTP functions both in vitro and in vivo in E. coli (Kaul et al., 1994; Young and Wake, 1994). It is generally believed by now that both terminator proteins mediate the replication fork arrest through specifically obstructing the replicative helicase operating ahead of the replication fork (Khatri et al., 1989; Lee et al., 1989). This activity has been termed “contrahelicase activity”. The Tus protein is able to inhibit a various (but not all) helicases including the SV40 large T-antigen (Hidaka et al., 1992). Interestingly, both the Ter-Tus and the Ter-RTP complexes are also able to arrest several RNA polymerases with the same polarity as replication forks (Mohanty et al., 1996; Mohanty et al., 1998).

The determination of the crystal structure of the RTP apoprotein at 2.6Å (Bussiere et al., 1995) revealed that it belongs to the winged helix family of proteins, and its closest structural homologue is histone H5. By contrast, the crystal structure of the Ter-Tus complex at 2.7Å (Kamada et al., 1996) revealed that the Tus protein has a unique overall fold and is structurally unrelated to other known protein structures, most notably RTP.

The physiological significance of the bacterial replication termini is not well understood yet. Since the tus and the rtp gene can be deleted without causing lethality, specific fork arrest and termination do not appear to be essential for cell viability (Hill et al., 1989). However, in both, E. coli and B. subtilis, fork arrest-systems have evolved
1 General Introduction

independently of one another. Thus, there is not much doubt that such a system must
confer a selective advantage. The Tus-Ter complex has been shown, for instance, to
prevent overreplication of plasmids carrying the E. coli oriC and two opposed Ter-sites
in vitro (Hiaasa and Marians, 1994). Another report suggests that the contrahelicase ac¬
tivity of the Ter-Tus complex prevents runaway replication in vivo (Krabbe et al.,
1997). Whereas the physiological role of the replication arrest system remains more or
less speculative, an interesting aspect of replication arrest has recently emerged. It
seems that homologous as well as illegitimate recombination can be triggered by ar¬
rested replication forks (Bierne et al., 1991; Horiuchi et al., 1994; Bierne et al., 1997),
for review see (Bierne and Michel, 1994).

1.2 Replication Termination in Eukaryotic Cells

In contrast to a bacterial genome, which is replicated by a single replicon, the
duplication of a eukaryotic genome is accomplished through many replicons. Thus,
there are numerous initiation and termination events, dispersed over the eukaryotic ge¬
nome during its replication. However, contrary to bacteria, sequence specific termina¬
tion seems to be the exception rather than the rule in eukaryotic organisms. It appears
that replication termination occurs wherever two converging replication forks of adja¬
cent replicons happen to meet, that is, within zones that span several kb (Hyrien, 1999).

In Saccharomyces Cerevisiae, analysis of a circular derivative of the chromo¬
some III that contains three origins of replication revealed three replication termination
zones that were located approximately midway between two neighbouring origins
(Greenfeder and Newlon, 1992). Altering the sites of replication initiation created a new
termination region, demonstrating that termination is not controlled by cis-acting se¬
quencies. Two other termination zones of Saccharomyces Cerevisiae have been exam¬
ined. A replication termination zone midway between two adjacent origins was found
on chromosome III (Zhu et al., 1992) and a broad termination zone was found cen¬
tromere-proximal to ARS501, which is a yeast origin of replication (Brewer and Fangman,
1993).
Termination of replication in the simian virus 40 (SV40) minichromosomes does not depend on specific sequences, it occurs wherever converging replication forks meet, about 180° around the circular genome from the origin (Brockman et al., 1975; Lai and Nathans, 1975). Occasional asymmetry in the rate of movement of the two diverging replication forks and the presence of sites where replication forks pause, lead to a zone of about 1kb, where replication terminates rather than a specific site located exactly 180° opposite the origin (Tapper and DePamphilis, 1980; Weaver et al., 1985). However, the sequences at the termination strongly influence the pathway, by which the two sibling molecules are separated (Fields-Berry and DePamphilis, 1989; Weaver et al., 1985).

There are only two well studied, sequence-specific replication termination sites currently known in eukaryotes. One is located in latent origin of replication of the Epstein-Barr Virus (EBV). The other one is located at the 3' end of rRNA genes, and exists probably in most, if not all eukaryotic organisms (Hernandez et al., 1993).

### 1.2.1 Site-specific replication termination in the Epstein-Barr Virus

The EBV can be maintained as a circular double-stranded, episomal plasmid of 172 kb in human cells by a latent infective cycle. A cis-acting element, the oriP, and a single trans-acting factor, the viral-encoded EBV nuclear antigen 1 (EBNA-1), are required for the virus' maintenance in human cells (Yates et al., 1984). The oriP is composed of two essential elements: the family of repeats (FR) that consist of 20 copies of a tandemly repeated 30 bp sequence, and, located about 1kb from the repeats, a dyad symmetry element (DS) (Reisman et al., 1985). Both, the DS and the FR encompass binding sites for the EBNA-1 protein, the FR one in every repeat. Replication of an 18 kb EBV plasmid, containing an oriP, has been studied in detail by 2-dimensional gel electrophoresis (Gahn and Schildkraut, 1989). Initiation of bi-directional replication occurs near or within the DS region. However, one of the replication forks is arrested at the FR, whereas the other fork traverses the circular plasmid to meet the arrested fork at the repeats, where the two replication forks merge and termination takes place. Thus,
the plasmid replicates predominantly in a unidirectional manner in human cells and both, replication initiation and termination, occur within the oriP.

The mechanism of the replication fork arrest was further dissected by reproducing the replication arrest in an SV40-based in vitro system using T-antigen and a cytosolic HeLa cell extract as a source of the replication proteins (Dhar and Schildkraut, 1991). A replication fork barrier was observed in the FR which was largely dependent on the addition of purified, recombinant EBNA-1 protein and also replication termination occurred at the repeats, as observed in vivo. Reducing the number of repeats from 20 to 6 had little effect on the efficiency of the barrier, but two repeats were not sufficient for stalling the replication fork.

In another in vitro study a series of truncated EBNA-1 proteins were investigated and it has been shown that the DNA binding and the dimerisation region of EBNA-1 is sufficient to elicit replication fork pausing (Ermakova et al., 1996). Furthermore, EBNA-1 bound to the FR inhibits DNA unwinding in vitro by the SV40 T antigen and DnaB helicase. In contrast to the Tus-Ter complex, however, this does not seem to occur in an orientation-dependent manner.

1.2.2 Site-specific replication termination in eukaryotic ribosomal genes

Probably the best studied eukaryotic replication termini and, to our knowledge, the only site-specific termini yet identified in the linear chromosomes of higher eukaryotes, are those at the 3' end of the rRNA genes (for review see (Lopez-Estrano et al., 1997), see also introductions 2.2 and 3.2 for further details). Initially, two groups discovered a permanent stalling of the replication fork moving in the direction opposite to transcription of the tandemly repeated 35S rRNA genes in Saccharomyces Cerevisiae. This permanent arrest of the replication fork has been termed replication fork barrier (RFB). The RFB is polar, that is, only replication forks moving in the direction opposite to transcription are stalled. Furthermore, it constitutes the first step of replication termination, because the replication fork of the adjacent replicon, travelling co-
directional to rRNA transcription, merges with the stalled replication fork, thus concluding replication.

At first glance, the most evident cause for the RFB would be the process of transcription itself, because the rRNA genes are heavily transcribed, even during the S-phase (Saffer and Miller, 1986). Moreover, the polarity of the RFB as well as its location close to the 3' end of actively transcribed rRNA genes (Lucchini and Sogo, 1994) would support this notion. However, by studying the replication of the *Saccharomyces Cerevisiae* rRNA gene cluster in the absence of ongoing transcription by polymerase I, it was convincingly shown that transcription elongation was not a prerequisite for the RFB (Brewer et al., 1992) (see also introduction 3.2 for a more thorough coverage of the relationship between the RFB and transcription). Furthermore, by transplanting sequences near the 3' end of the rRNA transcription unit into an episomal yeast plasmid, the minimal required sequence that elicits an RFB could be assigned to a 129 bp fragment (Brewer et al., 1992; Kobayashi et al., 1992). This sequence is located in the so-called nontranscribed spacer (NTS), which separates two adjacent rRNA transcription units and contains various regulatory elements (reviewed in (Reeder, 1989)). Interestingly, the 129 bp fragment essential for the RFB overlaps two other functional elements, one implicated in the enhancement of rRNA transcription (Elion and Warner, 1984; Elion and Warner, 1986) and one in mitotic recombination (Keil and Roeder, 1984; Voelkel-Meiman et al., 1987). This close spatial arrangement may suggest a functional relationship between these processes. Not only replication termination, but also replication initiation takes place within the NTS of yeast (Brewer and Fangman, 1988; Linskens and Huberman, 1988).

By now, RFBs have been identified in many disparate eukaryotic organism including humans (Hernandez et al., 1988; Little et al., 1993; Wiesendanger et al., 1994; Gerber et al., 1997; Lopez et al., 1998; Sanchez et al., 1998). There seem to be general features in the mode of replication of eukaryotic rRNA genes and the RFB seems to be a one of them (Hernandez et al., 1993). Whereas in general both, replication initiation and termination, take place in the NTS of eukaryotic rRNA gene clusters, an exception to this rule has been found in early *Xenopus* embryos, where replication forks move along the rRNA genes at a uniform rate and terminate at multiple, apparently random.
sites (Hyrien and Mechali, 1993; Hyrien et al., 1995). At this stage, however, the rRNA genes are not transcribed. By contrast, a polar RFB is found at the 3' end of the heavily transcribed rRNA genes in Xenopus cultured cells (Wiesendanger et al., 1994). A developmental regulation of the RFB along with the rRNA transcription in *Xenopus laevis* has recently been reported (Maric et al., 1999).

The molecular mechanism of the RFB remains largely elusive. There is strong evidence for the involvement of one or several trans-acting factors, possibly including a DNA binding protein. So far, two proteins have been implicated in the arrest of the replication fork at the RFB. The rRNA transcription termination factor TTF-I seems to be required for the murine and human RFB, as an *in vitro* replication approach has shown (Gerber et al., 1997). Interestingly, the yeast equivalent, Reb1p, seems to be dispensable for the yeast RFB activity, because its cognate binding site is not located in the DNA fragment essential for the RFB. TTF-I does not seem to be sufficient for the RFB, but rather a part of a larger protein complex that elicits the replication block at the RFB. Furthermore, the helicase activity of the T-antigen, which served as replicative helicase in the *in vitro* system, does not seem to be disturbed by TTF-I bound to its cognate DNA sequence (Gerber et al., 1997).

Genetic analysis has led to the identification of a yeast gene, FOB1, which is required for the stalling of the replication fork at the RFB as well as for the enhanced recombinational activity associated with sequences overlapping the DNA fragment essential for the RFB (Kobayashi and Horiuchi, 1996). Interestingly, deletion of the FOB1 gene does not exhibit any obvious growth defect, indicating that the yeast RFB, like the replication stalling at bacterial Ter-sites, is not required for the viability of the cell (Defossez et al., 1999). Recently, Fob1p has been implicated in the control of the rRNA gene number in yeast. It is essential for both decrease and increase of the rRNA gene copy number of yeast cells (Kobayashi et al., 1998). Furthermore, deletion of the FOB1 gene has been demonstrated to extend the life span of yeast mother cells (Defossez et al., 1999) by slowing down the generation of extrachromosomal rDNA circles, which in turn are a cause of ageing in yeast (Sinclair and Guarente, 1997).
1 General Introduction

The pleiotropic effect of FOB1 and the spatial arrangement of the regulatory elements in the yeast NTS suggest that the processes of replication, recombination and transcription are interwoven and share common regulatory factors.

1.3 Aim and Scope of the Work

The RFB is the only well-defined replication termination site currently known in the linear chromosomes of yeast and higher eukaryotes. This renders the RFB ideal for studying eukaryotic replication termination. Furthermore, there seems to be a functional relationship between replication arrest and the regulation of transcription, as well as processes of DNA recombination. In order to find out more about the still relatively obscure process of replication termination, and to understand the relationship of these processes better, we approached the topic on two different levels. As model organism the yeast Saccharomyces Cerevisiae was chosen, because it provides one of the most powerful genetic means, a sequenced genome and, last but not least, the RFB is best understood in this organism at present.

In one project we sought to determine the position of the arrested replication fork with the highest possible resolution, i.e. at nucleotide resolution. Furthermore, since the replication fork arrested at the RFB constitutes the first step of replication termination, we wanted to elucidate the architecture of the stalled fork, that is, the extension of the nascent leading and lagging strand as well as the processing status of the two nascent strands. This way, we sought to obtain information about the mechanism of replication termination or other processes in which the stalled replication fork is implicated, such as recombination.

A second project aimed at reconstituting the RFB in vitro. By this approach, we hoped to circumvent the complexity and technical limitation of an in vivo system, in order to dissect the molecular mechanism of the RFB and to identify factors that contribute to the replication arrest at the RFB.
2 THE ARCHITECTURE OF THE REPLICATION FORK STALLED AT 3' END OF THE YEAST RIBOSOMAL RNA GENES

2.1 Abstract

Every unit of the rRNA gene cluster of *S. cerevisiae* contains a unique site, where progressing replication forks are stalled in a polar manner termed replication fork barrier (RFB). By means of preparative two-dimensional (2D) gel electrophoresis, we isolated replicative intermediates (RIs) that were highly enriched for replication forks stalled at the RFB. The positions of the nascent strands at the RFB were determined at nucleotide resolution. We found a major arrest site overlapping the *Hpa*I restriction site at the border of a *Hpa*I-*Hind*III fragment essential for the RFB, and two minor, closely spaced arrest sites within this fragment. The discontinuously synthesised nascent lagging strand is extended three bases farther than the continuously synthesised leading strand. Furthermore, the majority of the stalled lagging strand was found to be completely processed. Thus, the stalled replication forks at the RFB hardly expose any single-stranded DNA on their newly synthesised arms. A model, explaining these findings in regard to the relative positions of the nascent strands, is presented. In order to get more information about the structure of the stalled replication forks, we have digested the RIs with T4 endonuclease VII, an enzyme recognising branched DNA molecules. We found that this enzyme cleaves predominantly in the newly synthesised, homologous arms, thereby specifically releasing the leading arm. Interestingly, no digestion products indicative of Holliday-like intermediates, a DNA structure recently proposed to occur at the RFB, were detected. This suggests that only very few, if any, of the stalled replication forks had adopted such a structure.
2 The Architecture of the Stalled Replication Fork

2.2 Introduction

In most eukaryotes, the rDNA is organised in a single or multiple clusters of a few hundreds to several thousands tandemly repeated units consisting of the heavily transcribed rRNA precursor genes separated by non-transcribed sequences, the non-transcribed spacers (NTSs) (Long and Dawid, 1980). A number of regulatory elements for virtually all processes of the DNA metabolism are present in the NTS. In _S. cerevisiae_, DNA replication initiates at an autonomous replication sequence (ARS), located in the NTS towards the 5' end of the rRNA transcription unit (see Figure 1), in about one every five to ten repeating units (Brewer and Fangman, 1988; Linskens and Huberman, 1988). The leftward moving replication fork travels through the NTS where it is stalled at the RFB in front of an active 35S gene (Lucchini and Sogo, 1994). The rightward travelling fork, moving in same direction as transcription, passes the rRNA genes unimpeded until it meets a replication fork stalled at the RFB, where the fusion of adjacent replicons takes place. As a result, the rDNA locus of almost all species investigated so far is replicated in a unidirectional manner.

The location of the stalled fork at the RFB in _S. cerevisiae_ was narrowed down to a 129 bp restriction fragment defined by a HindIII and HpaI restriction site (Figure 1) (Brewer et al., 1992). Furthermore, the sequences required for the block seem to reside within this fragment, even though additional sequences could be necessary to restore the full functionality of the RFB (Brewer et al., 1992; Kobayashi et al., 1992). An intriguing feature of the HindIII-HpaI fragment is its close proximity or overlapping with regulatory elements of transcription and recombination. Two transcription termination sites have been found just upstream of it and one within this fragment. The HindIII-HpaI fragment spans a part of the transcriptional enhancer element and overlaps with sequences required for the recombinational hotspot HOT1 (Elion and Warner, 1984; Keil and Roeder, 1984; Voelkel-Meiman et al., 1987).

The molecular mechanism of the RFB, however, still remains elusive. Initially, the replication fork arrest was attributed to head-on collisions of the replication machinery with RNA polymerase I (Brewer and Fangman, 1988), since the both processes have
been shown to take place simultaneously in the rRNA genes and because of the dense coverage of the rRNA gene by RNA polymerases I (Saffer and Miller, 1986).

However, it has been clearly demonstrated that transcription elongation is not a prerequisite for a functional RFB since a functional RFB was observed in a strain lacking the RNA polymerase I (Nogi et al., 1991; Brewer et al., 1992). Moreover, sequences close to the 3' end of the 35S rRNA gene retained their blocking ability when inserted into extrachromosomal plasmids lacking the transcription unit (Brewer et al., 1992; Kobayashi et al., 1992). On the other hand, these sequences did not impede replication fork movement on a plasmid in E. coli excluding possible structures inherent in the DNA sequence and suggesting the involvement of a yeast-specific protein binding to the RFB sequence and thereby impeding the movement of the replication fork, in analogy to the Tus-Ter system in E. coli (Brewer et al., 1992).

An attractive candidate for such a protein has been reported by Kobayashi and Horiuchi (Kobayashi and Horiuchi, 1996). Screening yeast mutants defective in HOT1 activity for the presence of an RFB, they found mutants lacking both, HOT1 and RFB, activities. Mutations in a previously uncharacterised protein with no homology to any other protein in the DNA data banks, termed Fob1p, were shown to be responsible for both defects (Kobayashi and Horiuchi, 1996). In this context it is noteworthy that fob1 mutants, in which replication of the rDNA is bi-directional, do not show any obvious growth defects (Kobayashi and Horiuchi, 1996). Because of the overlapping cis-elements for RFB and HOT1 activity and the pleiotropic effect of the fob1 mutation, a close link between the two activities has been proposed. This notion is supported by the observation that stalled replication forks at the Ter-sites of E. coli induce illegitimate and homologous recombination events (Bierne et al., 1991; Horiuchi et al., 1995). Further investigations have revealed that FOB1 is involved in the expansion / contraction of the rDNA repeats (Kobayashi et al., 1998) and that its elimination extends the life span of yeast mother cells by slowing down the generation of circular extrachromosomal rDNA circles (Defossez et al., 1999), which in turn have been shown to be a cause for ageing in yeast (Sinclair and Guarente, 1997). Additionally, Fob1 has been shown to be a nucleolar protein supporting the assumption that it could bind to the RFB region (Defossez et al., 1999).
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The stalled replication fork at the RFB seems to be a central intermediate in the processes of replication termination and recombination in the rDNA repeats. Furthermore, it is the only well defined replication termination site in the yeast chromosomes, making it a paradigm of eukaryotic replication termination.

Therefore, we strove to investigate the molecular structure of the stalled replication fork. We found that the architecture of the stalled replication fork is strikingly different from the current model derived from progressing replication forks, because it hardly exposes any single-stranded DNA and, surprisingly, the nascent lagging strand is extended three bp farther than the nascent leading strand. Moreover, no Holliday-like intermediates were detected, suggesting that the arrested replication forks rarely adopt such a structure.
2.3 Results

2.3.1 Isolation of replicative intermediates stalled at the RFB

We wished to explore the nature of the stalled forks at the RFB as precisely as possible. In a first step we sought to map the positions of the nascent strands at nucleotide resolution. Hence, an important prerequisite for our study was the availability of highly purified replicative intermediates (RIs) containing replication forks stalled at the RFB. The method we chose was preparative neutral/neutral 2D Gels (Lucchini and Sogo, 1994). In a first dimension the DNA molecules are subjected to a neutral agarose gel electrophoresis under conditions that predominantly separate DNA molecules according to their mass. Subsequently, the molecules are run at a 90° angle in another neutral agarose gel under conditions that separate DNA molecules mainly according to their shape (Brewer and Fangman, 1987). As a result, linear molecules migrate along a diagonal whereas replicative intermediates are retarded and migrate along specific, predictable patterns above the diagonal of the linear molecules. By digesting replicating rDNA with a restriction enzyme that cuts twice in the rDNA repeat, one cutting site located close to the rDNA autonomous replication sequence (ARS) element, mainly Y-shaped replicative intermediates will be generated. Such replicative intermediates migrate along an arc (the "Y-arc") as schematically depicted in Figure 1B. If a particular replicative intermediate accumulated as it occurs at replication pausing sites or replication fork barriers, a spot at its corresponding position on the otherwise smooth arc will appear.

CsCl enriched rDNA was digested with the restriction enzyme BgII that cleaves twice in the rDNA repeating unit giving rise to two fragments of equal length (see Figure 1A for a restriction map). The RFB, located in the centre of one of these fragments, leads to an accumulated Y-shaped RI with three arms of almost equal lengths migrating at the apex of the "Y-arc" (intense spot in Figure 1C). Because this is the most retarded point on the "Y-arc" (in respect to the diagonal of monomers) we considered the BgII digest to be ideally suited for the purpose of isolating highly purified RIs. In order to
isolate replication forks stalled at the RFB, a gel slice at the position of the intense spot at the apex of the "Y-arc" was cut out of the 2D gel (Figure 1D). A second gel slice was cut out at the position where the non-replicating, linear BglII fragments run (in this study referred to as the "monomers", 1n in Figure 1B). Subsequently, the DNA was recovered from the agarose slice. Closer inspection of an aliquot of the 2D gel purified RIs revealed that the purified DNA invariably consisted of three populations with a different electrophoretic mobility (Figure 1E). The major band with the slowest mobility represents intact replication forks stalled at the RFB as judged by its electrophoretic mobility, and because it is sensitive to T4 Endonuclease VII (see later). One of the two other bands shows the same migrating behaviour as does linear monomer DNA, whereas the other band migrates at a position expected for one of the three arms of the RI. We therefore assume that that the two faster migrating bands are due to breakdown products of replication forks, most likely resulting from shear breakage at the fork junction. The yield was assessed by comparing the signal intensities of the gel purified RIs with a dilution series of BglII digested rDNA repeats, subcloned into a plasmid (data not shown). Approximately 200 to 400 ng of intact replication forks were usually obtained per 2D gel.

It has been shown that exclusively leftward-moving forks are arrested at the RFB (Brewer and Fangman, 1988; Linskens and Huberman, 1988; Lucchini and Sogo, 1994). Therefore, we expected that the RIs we isolated consisted in the majority of stalled replication forks that had travelled leftwards. However, replication is initiated in only one of out of five or more rDNA repeating units (Walmsley et al., 1984; Brewer and Fangman, 1988; Linskens and Huberman, 1988). The other units are passively replicated by a rightward-moving replication forks. Consequently, the position of the RFB spot on the 2D gel is overlapped by a part of the "Y-arc" that consists of rightward moving replication forks. Such forks are inevitably co-purified with the leftward moving forks because of their similar mass and shape. However, since these replication forks are not stalled and therefore do not accumulate, they are under-represented in this population. But, in principle, these forks could interfere with subsequent analyses.
Figure 1: Purification replicative intermediates containing replication forks stalled at the RFB by preparative 2D gel electrophoresis. (A) Structural organisation and restriction map of the rDNA repeat unit of *S. cerevisiae*. The 35S precursor coding region and the sequences coding for the 5S rRNA are indicated as filled boxes, the ribosomal spacer as thin lines. The autonomous replication sequence (ARS) element and the RFB are also indicated. The positions of all restriction sites relevant for this work are indicated with respect to the 3' end of the 25S rRNA coding region (numbers in brackets) (Skryabin *et al.*, 1984). (B) Cartoon showing the migration behaviour of *BglII* digested replicating rDNA (only the fragment encompassing the RFB is depicted). Note that the Y-arc consists primarily of rightward-moving forks, whereas the spot of the accumulated RIs at the RFB consists of leftward-moving forks. The expected shortened bubble arc is not indicated. (C) rDNA from an exponentially growing yeast cell culture was digested with *BglII*, separated on a 2D gel, blotted, and hybridised with a probe encompassing the RFB region (indicated in Figure 3A, sketch 1, upper of the two probes). (D)
Southern blot of 2D gel used for purification of RIs stalled at the RFB (arrow 1) and linear monomers (arrow 2). The 2D gel conditions as well as the transfer and hybridisation procedure were exactly as in (C). Prior to the transfer, gel slices containing the accumulated RIs at the apex of the Y-arc and non-replicating monomers were cut out. (E) Southern blot analysis of the eluted RIs (1) and the corresponding linear monomers (2). The slowest migrating band of the eluted RIs in 1 represents the replication forks stalled at the RFB, the two faster migrating bands are probably the result of shear breakage at the fork junction giving rise to two linear molecules, one of them identical to the monomer except for a nick or gap. In some of the eluted of RIs the band co-migrating with the monomers was more intense than the faster migrating one, which indicates a contamination by monomers (see Figure 3A).
Figure 2. Analysis of the purified replication forks stalled at the RFB for the presence of rightward-moving forks. (A) rDNA restriction map spanning the BglII fragment encompassing the RFB. Fork 1 represents a leftward moving replication fork stalled at the RFB, fork 2 a rightward moving replication fork just passing the RFB site. These two forks cannot be fractionated on a 2D gel. To determine the fraction of rightward moving replication forks present in the 2D gel purified RIs an aliquot of the material re-digested with NheI. A leftward moving fork is cleaved in the unreplicated part of the molecule by NheI, giving rise to a Y-shaped molecules with almost twice the mass of the corresponding linear NheI digested BglII fragment. In contrast, the rightward moving fork is cleaved in its replicated part resulting in a molecule with a mass close to corresponding linear fragment. (B) Southern blot analysis of the 2D gel purified, BglII digested RIs that were re-digested with NheI and fractionated on a 1% agarose gel. After blotting, the BglII fragment encompassing the RFB was used as a probe. The asterisk in lane 2 indicates the position of the NheI cleaved, rightward moving replication forks. The band with the slowest mobility (arrow) represents NheI cleaved, leftward moving replication forks stalled at the RFB, whereas the fastest migrating band represents the small BglII-NheI fragment. The two bands immediately above are due to broken replication forks. Lane 4 shows the corresponding linear rDNA fragment digested with BglIII and NheI. Lanes 1 and 3 show aliquots of the RIs and monomers, respectively, before the NheI digest.
To assess the fraction of rightward moving replication forks present in the 2D gel purified DNA, we redigested the purified DNA with NheI, which cuts 460 bp upstream of the HindIII-HpaI fragment. Rightward-moving replication forks are cut in the newly synthesised arms. As a result, a Y-shaped DNA molecule consisting of a long stem and two short arms is generated. The mass of this molecule is close to a corresponding linear DNA fragment (Figure 2A). On the other hand, replication forks stalled at the RFB are cut in the unreplicated part giving rise to a Y-shaped DNA molecule with a short stem and two long arms with almost twice the mass of a corresponding linear DNA fragment. Southern blot analysis reveals that the vast majority of the purified RIs consists of replication forks stalled at the RFB (Figure 2, slowest migrating band in lane 2). At the expected position where the rightward-moving replication forks migrate on the agarose gel, only a weak and blurred band is detectable (indicated with an asterisk in lane 2). We conclude from this experiment that we have isolated highly purified replication forks stalled at the RFB accompanied by only a very minor contamination of rightward moving forks. These forks, however, should not interfere with subsequent analyses.

2.3.2 Analysis of the isolated RIs with the restriction enzyme HpaI

As a first step towards high resolution mapping of the RFB, we analysed the purified RIs with a restriction enzyme cutting close to the arrest site of the stalled replication fork. The branch point of the replication fork stalled at the RFB has been mapped to a position in the vicinity of the HpaI restriction site (Brewer et al., 1992; Kobayashi et al., 1992; Lucchini and Sogo, 1994). Theoretically, digestion of RIs with HpaI could give four different results depending on where the branch point of the fork was located in respect to the HpaI restriction site (schematically depicted in Figure 3A, sketches 1 to 4). A combination of these results is also conceivable, because the replication forks stalled at the RFB may not be homogenous in the position of the branch point. First, the replication fork could be blocked between the HindIII and HpaI site resulting in both, leading and lagging strand being cut by HpaI (sketch 1 in Figure 3A). In this case a
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fragment of about 1100 bp as well as a second, bigger than 300 bp would be generated. Second, still positioning the arrest site of the replication fork between the HindIII and HpaI restriction sites, the HpaI site could be located in a stretch of single-stranded DNA on the lagging strand. In this case, only the leading strand could be cut by HpaI and two restriction fragments of about 1400 bp and 1100 bp would arise (sketch 2 in Figure 3A). Third, the approaching replication fork could be blocked just a few basepairs before reaching the HpaI restriction site. The cut RIs would fall apart into linear DNA molecules, if the double stranded region sealing the two arms was short enough (sketch 3 in Figure 3A). Forth, the replication fork could be stalled before it reaches the HpaI restriction site. In this case a fragment between 1085 bp and 2170 bp would be observed (sketch 4 in Figure 3A).

2D gel purified RIs containing arrested replication forks and, as a control, equally purified monomers were first digested with EcoRI and Sphl (for restriction map see Figure 3A). Subsequently, an aliquot of the RIs as well as the monomers were digested with HpaI and analysed by Southern blotting. Inspection of the EcoRI-Sphl cut RIs prior to the HpaI digest clearly reveals that the isolated material does not only contain RIs (slow migrating band in lane 1 of Figure 3B) but also a monomer contamination that accounts for about 50% of the material (prominent faster migrating band, compare lane 1 with lane 4 in Figure 3B). This monomer contamination is probably due to overloading of the 2D gel with monomers (the monomer is in large excess over the RIs). However, these monomers do not interfere with the HpaI restriction analysis.

Digestion of the RIs stalled at the RFB with HpaI gives rise to four bands. Two of them appear in the digest of the monomers (compare lane 2 and 3 in Figure 3B). The two faster migrating bands (about 1100 bp and 300 bp) can be explained assuming that the RIs are digested as in depicted in sketch 3 or, alternatively, sketch 1 of Figure 3A. However, because the short fragment released from the RIs migrates at a very similar position as the 317 bp fragment of the HpaI digest of the monomers, the location of the branch point of the replication fork must be in the vicinity of the HpaI restriction site. Otherwise, if the branch point were located towards the HindIII restriction site, the short fragment of the RIs would be retarded due to its bigger mass and bulkier shape. This
suggests that the block may map close to the HpaI site. Digested contaminating monomers contribute to the presence of these two bands as well.

About 15% of the digested RIs give rise to a band migrating at 2100 bp (arrow in Figure 3B), which cannot be detected by a probe hybridising to the left of the HpaI site (compare lane 2 of Figure 3B with lane 6 of Figure 3C). This very reproducible band (three independent DNA preparations) can only be explained with the situation depicted in sketch 4 of Figure 3A, where the replication fork is stalled upstream of the HpaI site. A very faint signal (less than 5% of the total signal for the RIs stalled at the RFB) can be observed at a mobility of 1400 bp (indicated by an asterisk). The signal is also detected by the probe hybridising to the left of the HpaI site. This signal can be explained by the presence of a single-stranded stretch over the HpaI site on the lagging strand of the replication fork (Figure 3A, sketch 2). Taken together, the HpaI digest indicates that the majority of the 2D gel purified RIs consists of replication fork blocked close to the HpaI restriction site, and that there is virtually no stretch of single-stranded DNA on the lagging strand that overlaps the HpaI restriction site. A minority of the RIs however, consist of forks that are located downstream of the HpaI restriction site.
Figure 3. Hpal restriction analysis of replication forks stalled at the RFB. Symmetrical BgIII fragments of RIs (Figure 2A, fork 1) were re-digested with EcoRI and SphI generating a RI with a short non-replicated and two long replicated arms. (A) Theoretically possible cleavage of the replication forks stalled at the RFB by Hpal and the resulting fragments (for more detailed explanations see text): 1) The blocked replication fork is located between the HindIII and Hpal sites, Hpal cuts in both newly replicated arms. The black bars below the sketch of the replication fork denote the location of the probes used in (B) (upper bar) and (C), respectively. 2) As above, but the Hpal site is located in a single-stranded stretch on the lagging strand, Hpal cuts only in the leading arm. 3)
The blocked replication fork is located just a few basepairs downstream of the HpaI site. HpaI cuts just in front of the elongation point. The two newly replicated arms fall apart giving rise to two linear DNA molecules. 4) The replication fork is blocked before reaching the HpaI site, HpaI cuts in the unreplicated part of the RI giving rise to a linear and a branched DNA molecule. (B) Southern blot analysis of EcoRI-SphI digested RIs containing replication forks stalled at the RFB. The probe used for this blot is indicated in (A) and hybridises to both, the replicated arms as well as the unreplicated arm. The arrow in lane 2 indicates a slow migrating band that is due to replication forks located downstream of the HpaI restriction site (sketch 4 in 3A). The asterisk in lanes 2 and 6 indicates a minor band that could be due to RIs that were not cut in the lagging strand (sketch 2 in 3A). Note that the low intensity of the band at 300 bp is due to inefficient hybridisation, which inevitably occurs when the probe is longer than the detected strand. (C) same blot as in (B), stripped and re-hybridised with a probe located upstream of the HpaI site as indicated in (A, small bar).
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2.3.3 The nascent lagging strand is blocked at position 418, which is the first base of the *HpaI* restriction site and there are at least two minor block sites located towards the *HindIII* site

The *HpaI* digest indicates that the replication fork blocked at the RFB and the *HpaI* restriction site are in close vicinity. To obtain a higher resolution we carried out a PCR based primer extension assay. The experimental strategy of this assay is shown in Figure 4A. First, a 5' end-labelled primer is annealed to the replicated branches of the RIs. Subsequently, in a linear amplification reaction employing *Taq* polymerase, the primer is extended to the 5' end of the nascent lagging and the 5' end of the parental leading strand. The primer extension products are finally fractionated on a sequencing gel. Note that the result consists always of a composite pattern of the two template strands read by the *Taq* polymerase. Therefore, a primer extension of the isolated non-replicating monomers is always run in parallel as a control. Consequently, primer extension products that are only observed in reactions with RIs as templates can be assigned to the nascent lagging strand. Since RIs of replication forks stalled at the RFB are long-lived molecules in comparison to RIs of moving forks, we expected the majority of the Okazaki fragments on the lagging strand to be processed and ligated. Therefore, the mapped position of the 5' end of the lagging strand should coincide with the point of arrest of the lagging strand at the RFB. Note that this method cannot be used to map the point of arrest of the nascent leading strand at the stalled replication fork because of the polarity of the DNA.

*BglII* digested, 2D gel isolated RIs and monomers were used as templates for the extension of primer 580 (see materials and methods). The extension products were subsequently fractionated on a sequencing gel (Figure 4B, lane 2 and 5). As an internal control an aliquot of the RIs and monomers were digested with *HpaI* prior to the primer extension (lanes 1 and 4 in Figure 4B). Primer extension of RIs gives rise to a set of bands that do not appear in the primer extension of the monomer (compare lane 2 with lane 5 in Figure 4B). The most prominent of these bands denotes the major lagging strand block site of the stalled fork at the RFB. As expected from the results of the *HpaI* digest presented in Figure 3, the block site maps very closely to the *HpaI* restriction site,
namely to the G at position 418, the first base of the *HpaI* recognition sequence (compare lanes 1 and 2 in Figure 4B or, for a lower exposure, in Figure 4C) Interestingly, the restriction enzyme *HpaI* is still able to cut the RIs, despite of the close vicinity of the replication fork. There are a number of shorter primer extension products below the signal of the major stop. These signals, which account for about 20 to 30% of the total signal from the nascent lagging strand as quantified by Phosphoimager, could be due to approaching replication forks that have not yet reached the main stop. This notion is supported by the *HpaI* digest of RIs (Figure 3) where about 10 to 20% of the RIs seem to consist of forks that have not yet reached the *HpaI* site. An alternative explanation for these minor bands would be that they result from Okazaki fragments not yet fully processed.

There are two additional signals towards the *Hind*III site present in primer extension reaction of RIs at positions 330 to 340 (filled squares in Figure 4B, lane 2). These signals probably derive from two minor stop sites that have already been observed by Brewer et al. (Brewer *et al.*, 1992). Primer extension with primer 483, which 97 bp farther downstream than primer 580 (see materials and methods), led to the identical results (data not shown). Identical results were also obtained using yeast strain FTY23 (Thoma, 1986) instead of A1 (data not shown). We therefore conclude that the position of the block we mapped is not strain-dependant.
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(A) The architecture of the stalled replication fork is illustrated.

(B) Enzymes RFB and Monomer are shown with their respective digestion patterns.

(C) A close-up view of the RFB-Region is depicted.

(D) An example of the RFB with Vent and Tag markers.
**Figure 4.** Determination of the lagging strand arrest site at the RFB by primer extension. (A) Illustration of the strategy for mapping the lagging strand arrest site by primer extension (see text for details). (B) Autoradiogram of primer extension products fractionated on a 6% sequencing gel. Either RIIs (lanes 1 to 3) or monomers (lanes 4 to 6) were used as templates in the primer extension reaction. In lanes 1 and 4 the template DNA was digested with *HpaI* prior to the primer extension reaction, in lanes 3 and 6 treated with RNase H. Filled circles indicate the position of the major block site of the lagging strand, filled squares indicate the positions of minor lagging strand block sites, located towards the *HindIII* site. Lanes A, C, G, T show the corresponding sequencing ladder generated by primer extension using the same primer and CsCl purified rDNA as template in the presence of ddATP, ddCTP, ddGTP and ddTTP, respectively. (C) enlargement of a part of the same sequencing gel as in (B), showing a shorter exposure of the signal for the major lagging strand block site. (D) Autoradiogram of sequencing gel showing the products of two primer extension reactions that were identical, except for the DNA polymerase. In one reaction Taq polymerase was used to elongate the primer, whereas in the other Vent (exo-) was used for this purpose. In these conditions, optimised for the Taq polymerase, the efficiency of the Vent (exo-) was lower than that of the Taq polymerase.
2.3.4 The stalled nascent lagging strand at the RFB is devoid of the RNA primer

Next, we wished to find out whether we mapped the 5' position of the last RNA primer on the lagging strand or whether the RNA primer was already processed at the stalled replication fork. Taq polymerase is known to possess a weak reverse transcriptase activity (Newton and Graham, 1994). For this purpose, we digested an aliquot of the RIs with RNase H, which specifically digests RNA in DNA-RNA hybrid, prior to the primer extension reaction. The resulting primer extension products were identical to those where untreated RIs were used as templates (compare lanes 2 and 3 in Figure 4B). This result indicates that we mapped the 5' DNA end of the nascent lagging strand. However, since we do not have a positive control for the RNase H, we performed an additional primer extension using another thermostable DNA polymerase, the Vent (exo-) polymerase, and compared the products with the ones of the Taq polymerase. The Vent (exo-) polymerase is known to be unable to use RNA as a template and therefore only reads to the DNA-RNA junction of a RI (Bielinsky and Gerbi, 1998). Both DNA polymerases gave rise to virtually identical primer extension products, except that the product of the major stop site seems to be one base shorter when elongated with Vent (exo-) polymerase (Figure 4D). However, this difference results most likely from the terminal transferase activity of the Taq polymerase, which is much weaker in the case of the Vent (exo-) polymerase (Newton and Graham, 1994). If the Taq polymerase was extended until the 5' end of the last RNA primer, the product would be approximately 10 bp longer than the one of the Vent (exo-) polymerase, because the average length of an RNA primer used for initiation of Okazaki fragment synthesis in eukaryotes has shown to be about 10 bp (Burhans et al., 1991). Clearly, we have not mapped the 5' end of such an RNA primer. On the other hand, we cannot rule out the possibility that the RNA primer is present but not detectable in our assay. The Taq polymerase could have stopped at the DNA-RNA junction on the nascent lagging strand. However, in this case the RNA primer would extend over the HpaI restriction site, making cleavage of the lagging strand by HpaI impossible. The results of the HpaI digestion shown in Figure 3 clearly contradict such assumptions. Therefore, we conclude that the Okazaki
fragments on the lagging strand of the replication fork stalled at the RFB are fully processed and the last RNA primer is removed after the replication fork has reached the RFB.

2.3.5 The nascent leading strand and the nascent lagging strand are stalled at a position very close to each other

Since the elongation point of the nascent leading strand cannot be mapped by primer extension, we had to devise another method to map the arrest site of the leading strand. In E. coli, the arrest site of the leading strand at the Ter site was mapped by end-labelling RIs of blocked replication forks with T4 polynucleotide kinase (Mohanty et al., 1998). However, with this method, it would be difficult to distinguish between the nascent leading and lagging strand, if they were of similar length. Therefore, we decided to use an assay loosely related to an indirect end-labelling assay. First, the purified RIs blocked at the RFB were digested with SnaI, which generates a 757 bp DNA fragment encompassing the RFB. The fragments were then fractionated on a denaturing 6% acrylamide, 7M urea gel and subsequently electro-transferred onto a Nylon membrane. Finally, the membrane was hybridised with a strand-specific probe close to the SnaI restriction site (Figure 5A). A probe specific for the top strand will detect the parental leading as well as the nascent lagging strand, a probe specific for the bottom strand the parental lagging and the nascent leading strand. The signals for the parental strands will be detected at the position of 757 bp, the nascent strands will be shorter. Therefore, the precise location(s) of the nascent strands can be deduced from their size. To identify potentially interfering bands from nicked DNA, non-replicating, SnaI cut DNA, was run in parallel as a control.

Figure 5B shows a Southern blot with nucleotide resolution of SnaI digested replication forks stalled at the RFB that were fractionated on a sequencing gel. The membrane was first hybridised with a probe specific for the top strand (lanes 1 to 3). The membrane was subsequently stripped and re-hybridised with a probe specific for the bottom strand (lanes 4 to 6). As a background control, non-replicating monomers
were treated exactly the same way as the RIs and were run in parallel (Figure 5B, lanes 2 and 5). Additionally, *HpaI* digested monomers were run on the same gel as an internal size marker (Figure 5B, lanes 1 and 4). As expected, the strand-specific probe for the nascent lagging strand detects the major arrest site of the stalled replication fork at position 418, close to the *HpaI* site (indicated by a filled circle in Figure 5B), as well as the two minor arrest sites towards the *HindIII* site (filled squares in Figure 5B). These results are in good agreement with the mapping of the blocked replication fork at the RFB by primer extension (Figure 4).

The probe specific for nascent leading strand shows a major arrest site in the vicinity of the *HpaI* restriction site, very close to the position of the lagging strand block site. Unexpectedly, the major arrest site of the leading strand is located three bases downstream of the lagging strand, at the position 421 (open circle in Figure 5B on the right). Additionally, there are at least two minor arrest sites, mapping to positions close to the ones of the minor lagging strand blocks (open squares Figure 5B on the right). However, two more bands appear at positions at which no corresponding bands for the lagging strand are present (indicated by asterisk in Figure 5B). The slower migrating one of them would map to a position outside of the *HpaI-HindIII* fragment and is therefore unlikely to be the product of a stalled replication fork at the RFB. However, the faster migration band could be interpreted as the product resulting from an additional arrest site of the leading strand. The corresponding nascent lagging strand could either be blocked at position 418 bp thereby exposing a 85 bp stretch of single-stranded or it could be blocked at one of the minor arrest site. In the first case, a fraction of the replication forks stalled at the RFB would expose a stretch of single-stranded DNA, which contradicts the results of the *HpaI* analysis of the RIs presented in Figure 3.

We conclude from these data that the majority of the leading and lagging strand of the stalled replication fork at the RFB are extended to very similar positions with the nascent lagging strand being three bases ahead of the nascent leading strand (for details see Figure 6).
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(A) 35S RFB

(B) Lagging Leading Stand
M/H M R M/H M R

(C) Lagging Strand Leading Strand
H/M M R H/M M R

1 2 3 4 5 6
Figure 5: Mapping of the arrest site of the leading strand by Southern blot analysis of a 6% sequencing gel. (A) Restriction map showing the 757 bp SnaI fragment encompassing the RFB. A replication fork stalled at the RFB and the corresponding monomer are shown below. The replication fork consists of two long parental strands of 757 bp and of two short nascent strands, the nascent leading strand, depicted as an arrow and the nascent lagging strand, depicted as a line. The nascent strands can be detected individually on a Southern blot by using strand-specific probes that hybridise to sequences close to the SnaI restriction site at the right end of the fragment. The probe detecting the nascent leading strand (grey rectangle) will also detect the parental lagging strand, whereas the probe detecting the nascent lagging strand (black rectangle) will also detect the parental leading strand. As a background control and as internal length standard, SnaI and SnaI-HpaI digested monomers, respectively, were run in parallel. In contrast to the RIs, both strand-specific probes should give rise to the same signal for the monomers (both, SnaI and HpaI, generate blunt ends). (B) RIs containing replication forks stalled at the RFB and the corresponding monomers were digested with SnaI and, in case of the monomer, with SnaI and HpaI. The DNA was purified and subsequently fractionated on a 6% sequencing gel. After the electrophoresis, the gel was cut into an upper and lower part of similar size and electro-blotted onto a nylon membrane. The sequencing lanes, generated by extension of a 5'-endlabelled primer, were cut off after the transfer. The rest of the membrane was sequentially hybridised with a strand-specific probe detecting the nascent lagging strand (lanes 1, 2 and 3) and with a strand-specific probe detecting the nascent leading strand (lanes 4, 5 and 6). Note that the fragments of the restriction digests and the shown sequences cannot be directly aligned, because the primer did not anneal at the SnaI site. The circles indicate the major block sites for the leading and lagging strand, respectively. The squares indicate minor stop sites of the strands towards the HindIII site. Asterisks indicate bands that are detected only with the probe specific for the nascent leading strand. M/H: Monomers digested with SnaI and HpaI. M: Monomers digested with SnaI. R: RIs containing replication forks stalled at the RFB digested with HpaI. (C) Enlargement of the part of the Southern blot showing the major block site of the lagging strand as well as the corresponding site of the leading strand.
Figure 6: Nucleotide sequence of the HindIII-HpaI fragment and downstream flanking region. The filled circle indicates the mapped position of the nascent lagging strand and the open circle the position of the nascent leading strand of the stalled replication fork at the RFB. The rectangles show the two minor stop sites toward the HindIII site. The block may not be exactly at the indicated nucleotide but rather over the region indicated by the bracket.
2.3.6 T4 endonuclease VII discriminates between the leading and lagging strand

There are a number of DNA modifying enzymes specific for branched DNA molecules (for reviews see (White et al., 1997; Kemper, 1997)). Such enzymes could be useful tools to characterise and analyse RIs in combination with the primer extension assay described in this study. T4 endonuclease VII (endo VII), one of these enzymes, is exceptional in its ability to use a large number of branched DNA structures and/or structural perturbations in DNA as substrates (reviewed in (Kemper, 1997)). These include, among others, three way junctions (Y-structures) as occur at the replication fork during DNA replication. Endo VII has been shown to resolve a variety of synthetically constructed Y-structures into linear molecules by introducing nicks 3′ to the junction (Jensch and Kemper, 1986; Kemper et al., 1990; Pottmeyer and Kemper, 1992). To our knowledge, however, endo VII cleavage of naturally occurring RIs has not been investigated so far. A major difference between the naturally occurring RIs and the synthetic Y-structures are the two homologous arms of RIs because the synthetic Y-structures investigated so far consisted of three non-homologous arms.

To resolve RIs into linear DNA molecules at least one nick has to be introduced in the vicinity of the branch point in either the parental leading or the parental lagging strand. These nicks can be detected by primer extension. A primer annealing to the un-replicated part of a given RI detects the introduced nicks in the parental lagging strand, one annealing to the replicated arms, detects the nicks in the parental leading strand (Figure 4A, but also Figure 7B and C). Note that the primer extension products of the parental leading strand will be accompanied by the products of the nascent lagging strand.

BglII digested, 2D gel purified rDNA consisting of about 50% RIs and 50% linear monomers (not shown) were re-digested with EcoRI and SphI (Figure 7A, lane 1) in order to produce asymmetrical RIs with a short, unreplicated and two long, replicated arms (Figure 7A, lower panel). Subsequently, the RIs and monomers were treated with increasing amounts of endo VII (Figure 7A). 200 U of endo VII were sufficient to resolve the Y-shaped RIs completely (lane 4, Figure 7A). The retarded band of the RIs
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has disappeared completely giving rise to two faster migrating products: a band co-migrating with the linear monomers and a shorter one, migrating at the size of a replicated arm of the RI. This suggests that one of the replicated arms was released. In short, this experiment indicates that, at a time, only one of the replicated arms, either the leading or the lagging arm, is cleaved by endo VII (lower panel of Figure 7A).

This experiment, however, does not give us any clue about which strand is released by endo VII. Does endo VII cut randomly in the leading and the lagging strand resulting in 50% released leading and 50% released lagging strand? Is there a preference for one of the strands or does endo VII release only one strand, thereby cleaving either leading or lagging strand? To answer these questions we employed the primer extension assay using aliquots of the RIs digested with 200 U endo VII as templates.

First, primer 256 (see materials and methods), which anneals to parental lagging strand in the unreplicated part of the RIs, was elongated and cuts in the lagging strand are detected. The primer extension products were fractionated on a denaturing polyacrylamide gel and the products of reactions with endo VII treated RIs were compared to the products of reactions with untreated RIs. No bands indicative of endo VII cleavage in the parental lagging strand were observed (compare lanes 1 and 2 in Figure 7B). This result strongly implies that endo VII degrades the RIs by cutting exclusively in the parental leading strand.

In a next step, we carried out a primer extension using primer 580 (see materials and methods), that anneals to parental lagging strand in the unreplicated part of the RIs, was elongated and cuts in the lagging strand are detected. The primer extension products were fractionated on a denaturing polyacrylamide gel and the products of reactions with endo VII treated RIs were compared to the products of reactions with untreated RIs. No bands indicative of endo VII cleavage in the parental lagging strand were observed (compare lanes 1 and 2 in Figure 7B). This result strongly implies that endo VII degrades the RIs by cutting exclusively in the parental leading strand.
endo VII. This seems plausible, because the incisions in the nascent lagging strand seem to occur over maximally 11 nucleotides and are 3' to the junction. Endo VII has been shown to cut over 2 to 6 nucleotides 3' to the junction (Pottmeyer and Kemper, 1992). However, the observed signals close to the *HpaI* site in endo VII treated RIs must also be due to cleavage in the parental leading strand, because this strand is released by endo VII treatment. Since the nascent leading strand maps to a position of three nucleotides behind of the branch point, as shown in this study, incisions 3' to branch point on the parental leading strand give rise to very similar products as the ones from the nascent lagging strand. This is what we actually observe. Taken together, our data strongly suggests that endo VII cuts exclusively in the homologous, replicated arms of RIs (as depicted in Figure 7C, lowest panel). As a consequence, the leading arm of an RI can be selectively removed with the help of endo VII.
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(A) T4 Endonuclease VII

(B) Cut in Parental Leading Strand
No Cut in Parental Lagging Strand

(C) A G C T + + Endo VII

Unreplicated Arm

Cut in Parental Leading
Cut in Nascent Lagging Strand

RFB

EcoRI

SphI

HindIII

R

M

Hpal

1 2 3 4
Figure 7: Endo VII digestion of replication forks stalled at the RFB specifically releases the leading strand of the RI. (A) 2D gel purified RIs stalled at the RFB and linear monomers were digested with EcoRI and SphI, treated with endo VII and the products were analysed on a Southern blot using the same probe as in Figure 3B. A mixture of about 50% RIs and 50% monomers was incubated for 30 minutes at 37°C with 0U, 10U, 50U and 200U endo VII, respectively (lanes 1, 2, 3 and 4, respectively). Approximately the same amount of monomers only was incubated under the same conditions (lanes 5 to 8). 200U endo VII are sufficient to resolve the RIs completely into two linear DNA molecules (see text for details). Lower panel: schematic diagram of the EcoRI-SphI fragments containing the stalled replication fork at the RFB and the resulting products of the endo VII digest; either the leading strand or the lagging strand of the RI are released by endo VII. (B) Primer Extension analysis of the parental lagging strand of the replication forks stalled at the RFB. If the lagging strand of the RIs were released upon treatment with endo VII, a nick would have been introduced into the parental lagging strand by endo VII, which in turn would be detected by this primer. Upper panel: Autoradiogram showing primer extension products fractionated on a 6% sequencing gel. RIs treated with (lane 1) or without endo VII (lane 2) or monomers treated with (lane 3) or without endo VII (lane 4) were used as templates. Lower panel: Schematic representation of a RI showing the annealing position of the primer as well as the presumed cutting sites of the endo VII (see text for details). (C) Upper panel: Autoradiogram of sequencing gel showing the products of primer extension reactions with the same templates as in (B), but a primer annealing to the replicated arms. RIs treated with and without endo VII are shown in lane 1 and 2, respectively, the corresponding equally treated monomers in lane 3 and 4, respectively. On the right side, an enlargement of a part of the sequencing gel is shown. Differences in the primer extension products of RIs treated with and without endo VII are clearly visible. The panel in the middle shows the annealing positions of the primer and the presumed cutting sites of endo VII. The lower panel illustrates the assumed symmetrical cleavage in the homologous, newly replicated arms.
2.4 Discussion

In the present work, we have analysed the structure of the arrested replication fork at the RFB at nucleotide resolution. By means of preparative 2D gel electrophoresis we were able to isolate a population of DNA molecules consisting almost exclusively of replication forks stalled at the RFB. Primer extension analysis of the nascent lagging strand revealed a major arrest site at position 418 and at least two minor arrest sites towards the 3’ end of the rDNA transcription unit, at positions 330 to 340. The Okazaki fragments on the lagging arm are fully processed with no RNA primer being attached to the last Okazaki fragment. An indirect end-labelling assay revealed that the major arrest site of the leading strand is located at position 421, three bases behind the nascent lagging strand. Thus, the replication fork stalled at the RFB hardly exposes any single-stranded DNA on its newly synthesised arms. Finally, by digestion of the isolated replication forks with endo VII, we were able to specifically release the leading arm from the RIs.

2.4.1 The location of the major and minor arrest sites of the stalled replication fork at the RFB

The location of the RFB has previously been mapped at low resolution by two independent assays: to a 129 bp fragment between the HindIII and HpaI restriction sites (position 286 to 415, see Figure 1) by means of neutral/neutral 2D gel electrophoresis (Brewer et al., 1992; Kobayashi et al., 1992) and to position 376 ± 70 bp by electron microscopy (Lucchini and Sogo, 1994). Moreover, the minimal sequences required for the block of the replication fork seem to reside within this fragment (Brewer et al., 1992; Kobayashi et al., 1992). We mapped the major arrest point to a position three bases in front of the HpaI cutting site, overlapping the first nucleotide of its recognition sequence. Even though the block we mapped is not located within the 129 bp HpaI-HindIII fragment, our result is coherent with the previous mappings, because, as long as the HpaI restriction enzyme is still able to cut the DNA, the two arms of the stalled rep-
lication will be released and the spot of the accumulated RIs will disappear on the 2D gel upon \textit{HpaI} digestion. It is interesting to note that \textit{HpaI} retains its full cleavage ability despite of the partial overlapping replication fork. Since \textit{HpaI} may need a completely double-stranded restriction site, it is possible that the restriction enzyme forces the replication fork into a conformation, in which a full double-stranded recognition sequence is restored. It has been reported that an \textit{EcoRI} restriction site, located across the junction of an artificially constructed Y-structured DNA molecule, was cut to completion by the restriction enzyme (Jensch and Kemper, 1986), perhaps by a similar mechanism.

Because of its close vicinity to the stalled replication fork at the RFB, the \textit{HpaI} digestion provided us with valuable information about the structure of the isolated replication forks. Digestion of the isolated RIs with \textit{HpaI} resulted in a slower migrating band contributing to 10 to 20\% of the isolated RIs that can be explained by the presence of replication forks located in front of the \textit{HpaI} restriction site. This notion was confirmed by the primer extension analysis, where a series of bands below the major signal of the 5' end of the nascent lagging strand appeared. These replication forks could be due to various minor arrest sites in front of the major arrest at position 418, or, alternatively, of approaching replication forks that are just about to reach the arrest point. We favour the latter interpretation. The presence of such approaching forks in the gel-isolated material is to be expected considering the low resolution of agarose 2D gel electrophoresis. The relatively high contribution of 10 to 20\% to the long-lived stalled replication forks at the RFB could be indicative of a slowing-down of the replication forks in front of the RFB and the pattern of the primer extension product could reflect individual Okazaki fragments.

Upon close inspection of the spot due to the accumulated replication forks stalled at the RFB Brewer et al. (Brewer \textit{et al}., 1992) observed a somewhat elongated signal that appeared to consist of two discrete spots of different intensities. The origin proximal spot was more intense and most likely coincides with the major block we have detected at position 418. Likewise, we have observed at least two closely spaced minor block sites, about 90 bp closer towards the \textit{HindIII} site than the major arrest site. The position and the intensity of them strongly suggests that these two stops correspond to
the other, weaker spot observed by Brewer et al. Whereas at least two stops are detected by primer extension, only one will be detected by 2D gel electrophoresis, because the two arrest sites are too closely spaced to be resolved. It still remains to be elucidated whether the major and minor stop sites are alternative arrest site or whether a given replication fork is sequentially stalled at the major and one or both minor stop sites (Brewer et al., 1992). In the first case, less replication forks would be stalled at the minor block sites, whereas in the second case, the replication fork would be blocked for a shorter period at the minor sites.

2.4.2 The processing of the lagging strand

Primer extension of the RIs stalled at the RFB did not give rise to a population of primer extension products distributed over a broad region, but rather to a single, predominant product. This finding indicates that the majority of the replication forks stalled at the RFB are fully processed. Since the stalled replication forks at the RFB are long-lived RIs that remain halted at the RFB up to several minutes (assuming that the speed of the fork movement is constant and approximately 50 bp/s), there is sufficient time to complete the processing of the last Okazaki fragment. In the SV40 system, the maturation of the Okazaki fragment is known to take about one minute (DePamphilis and Wassarman, 1980). Furthermore, since the fusion of two adjacent replicons in the rDNA takes place at the RFB, it seems that the stalled nascent lagging strand does not need further modification before the ligation with the nascent leading strand from the opposite moving fork.

2.4.3 Similar position of the nascent strands at the RFB

A remarkable feature of the stalled replication fork at the RFB in *S. cerevisiae* is its lack of a single-stranded DNA on the newly synthesised lagging arm. Besides the data presented in Figure 5, there are three additional lines of evidence indicating that the stalled replication fork at the RFB does not expose a significant stretch of single-
stranded DNA on the lagging strand. First, when RIs were enriched by BND cellulose, a considerable amount of the accumulated RIs from the RFB eluted together with the non-replicating, linear DNA molecules in the "salt wash" (Linskens and Huberman, 1988) and data confirmed in our group). This indicates that the blocked replication forks at the RFB expose less single-stranded DNA than progressing replication forks. Second, in a study of the chromatin structure of replication forks stalled at the RFB by electron microscopy, the DNA immediately behind the forks appeared mostly double stranded (Lucchini and Sogo, 1994). Finally, the *HpaI* digest presented in this study suggests that the leading strand is not extended beyond the *HpaI* restriction site, because in this case *HpaI* could not cleave in the single-stranded lagging arm.

Another salient feature of the stalled replication fork at the RFB is that the 5' DNA end of the processed nascent lagging strand is located three bases ahead of the 3' end of the nascent leading strand. This finding raises intriguing questions about the molecular mechanism of the replication fork block at the RFB. We propose a model exploiting the asymmetry of the replication fork to explain this finding (Figure 8A): Contrary to the continuously synthesised nascent leading strand, the nascent lagging strand is known to be synthesised discontinuously in the direction opposite to the replication fork movement by Okazaki fragments, in eukaryotes of 40 to 300 bp in size (DePamphilis and Wassarman, 1980). These fragments can only be synthesised by elongation of an initiator DNA (iDNA), consisting of a RNA-DNA primer generated by the DNA polymerase δ (pol δ) / primase (Nethanel et al., 1992; Waga and Stillman, 1994). The iDNA has an average length of about 40 bp of which about 10 bp consist of RNA. Thus, the nascent leading strand was blocked at least 10 bp ahead of the 5' end of last primer of the lagging strand.

Recent advantages in the enzymology of the replication fork have revealed the basic architecture of the eukaryotic replication fork (for reviews see Bambara et al., 1997; Waga and Stillman, 1998). A key enzyme of the replication process is the replicative helicase, which operates ahead of the DNA synthesis, promoting the unwinding of the DNA duplex. Currently, the yeast replicative helicase has not yet unequivocally identified. However, there is evidence suggesting that a complex of three MCM proteins (Mcm 4, 6, and 7) functions as a replicative DNA helicase at the yeast replication fork.
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(Aparicio et al., 1997; Ishimi, 1997). The MCM helicase tracks 3' to 5' on the DNA, thus using the parental leading strand as template strand (Ishimi et al., 1998). Upon encountering the RFB from the nonpermissive direction, the replication fork will be arrested, possibly by a DNA binding protein inhibiting the replicative helicase, similar as proposed for the bacterial replication arrest at the Ter-sites (Khatri et al., 1989; Lee et al., 1989). The lagging arm will expose a stretch of single-stranded DNA up to the unwinding point, stabilised by a ssDNA-binding protein (RP-A), whereas the replicative helicase occupies a part of the unwound parental leading. As a result, the nascent leading strand not being extended up to the unwinding point (Figure 8A, sketch 1). The polα/primase complex will prime DNA synthesis by synthesis of an iDNA on the lagging template, close to the unwinding point and ahead of the 3' end of the nascent leading strand (Figure 8A, sketch 2). Subsequently, the gap to the penultimate Okazaki fragment will be filled, either by the polα/primase complex itself or by the polymerase synthesising the bulk of the lagging strand, possibly polα (Figure 8A, sketch 3). Finally, the RNA primer will be removed with the help of a helicase and nucleases (Bambara et al., 1997).

As a result, the lagging strand would be extended to a position ahead of the leading strand (Figure 8A, sketch 4). Whether the placement of the 5' end of the iDNA ahead of the 3' growing end of the nascent leading strand, a salient feature of this model, is a general mechanism of the DNA replication process or a peculiarity of the replication fork block at the RFB, remains to be determined.

This model would also explain the relatively homogenous placement of the Okazaki fragments giving rise to major signal at position 418.

An alternative model could involve a replication fork blocking activity interfering only with the synthesis of the leading strand at first. This could lead to an uncoupling of the lagging strand synthesis similar as proposed for replication fork bypass of a lesion located on the leading strand (Cordeiro-Stone et al., 1997). The lagging strand would be elongated farther until it would also be stalled by some replication fork blocking activity, which could be located at a similar position as the block of the nascent leading strand. The salient feature of this model is two separable blocking activities for the nascent leading and the nascent lagging strand.
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A) Figure 8: Possible events occurring at the stalled replication fork. (A) Model illustrating the processing of the nascent lagging strand of an arrested replication fork at the yeast RFB. 1) Schematic drawing of a progressing replication fork shortly before reaching the RFB. Only a selection of the proteins involved in DNA replication is depicted. The replicative helicase, moving in 3' to 5' direction along the parental leading strand, promotes DNA unwinding at the replication fork. The bulk DNA synthesis is carried out by polymerase $\delta$ / PCNA on the leading and, possibly, on the lagging strand. In general, the growing point of the leading strand is ahead of the synthesised nascent lagging strand. The single-stranded stretches on the parental lagging strand are covered with RP-A, a single-strand binding protein (not depicted). 2) The replication fork is stalled, possibly by a DNA binding protein (may be Fob1p). The nascent leading stand comes to a stop. At the same time an i-DNA primer is synthesised on the single-stranded stretch of the

B)
parental lagging strand by the polσ / primase complex. Because the helicase moves along the parental leading strand, the primer is synthesised ahead of the growing point of the nascent leading strand. 3) The gap to the penultimate Okazaki fragment is filled. This might be accomplished by the polσ / primase complex or, as depicted here, by the polymerase synthesising the bulk of the lagging strand. 4) Removal of the primers and ligation of the nick on the nascent lagging strand results in a replicative intermediate where both nascent strand are extended to a similar position. Thus, the nascent lagging strand can be extended farther than the nascent leading strand, which is what we have found at the yeast RFB. Whether the proteins belonging to the replication machinery remain bound, as depicted here, or come down as soon as the processing of the nascent lagging strand is finished, has to be elucidated still. (B) Schematic representation of the postulated Holliday-like DNA structure for the stalled replication forks at the RFB. Arrows indicate the potential cleavage sites. Homologous arms are depicted in grey.
2.4.4 The stalled replication fork, Fob1 and recombination

The overlapping of sequences essential for replication fork blockage at the RFB and HOT1 dependent mitotic recombination suggests a relationship between the two events. This notion was corroborated by the identification of the FOB1-gene, which is essential for both, HOT1 and RFB, functions (Kobayashi and Horiuchi, 1996). Furthermore, Fob1 has shown to be essential for the expansion and contraction of the rDNA repeats and plays a role in the generation of ERCs (Defossez et al., 1999; Kobayashi et al., 1998). For both processes, a model involving the stalled replication fork as a substrate for a recombination process has been proposed. Kobayashi et al. propose a double strand break (DSB) by nuclease cleavage of an exposed single-stranded region and subsequent repair by gene conversion.

In general, replication forks halted for a prolonged time seem to be recombinogenic (reviewed in (Bierne and Michel, 1994)). In E. coli, for instance, blockage of the replication fork at Ter-site has been shown to stimulate homologous recombination (Horiuchi and Fujimura, 1995; Horiuchi et al., 1994) and the Ter-site proved to be a strong deletion hotspot (Bierne et al., 1991). There are several potential causes for a connection between these two processes including exposed single-stranded DNA on the lagging arm of the replication fork. We have found strikingly little single-stranded DNA exposed by the stalled replication fork. Whether this has any functional significance for the relationship between the replication fork blockage and recombinational events remains to be determined.

A model taking advantage of recent findings in E. coli (Seigneur et al., 1998), which provide a mechanistic link between replication arrest and homologous recombination, has recently been proposed by Defossez et al. A central intermediate in this model is a DNA structure resembling a Holliday junction (Figure 8B, see also (Defossez et al., 1999), generated by the pairing of the two nascent strands. If an accumulation of such a structure was present in the RIs we isolated, we should have detected it by endo VII digestion and subsequent primer extension, because endo VII specifically resolves holiday junctions (Mizuuchi et al., 1982). Nicks would be introduced two to six bp 3' to the junction (Pottmeyer and Kemper, 1992), mainly on the homologous
arms presumably (Lilley and Kemper, 1984), that is, on the parental leading and nascent lagging strand (lower panel of Figure 7). However, since we have only found primer extension signals within a stretch of maximally 11 bp from the branch point of the replication fork (Figure 6C), the annealed nascent strands must be either only few bp long (less than 11 bp) or not abundant enough to be detected in our assay. Another possibility is that we have lost these structures during the 2D gel isolation of RIs. However, during the gel-isolation procedure the RIs were heated to 65°C, which might even promote the annealing of the nascent strands.

If Holliday-like DNA structures were an intermediate that a significant number of the stalled replication forks adopted, it should be possible to capture these molecules by EM analysis of psoralen crosslinked rRNA genes. Psoralen is a DNA-intercalating drug that “freezes” DNA structures and prevents branch migration (Schwacha and Kleckner, 1997). Therefore, we have scrutinised psoralen crosslinked replication forks stalled at RFB for the occurrence of Holliday-like DNA structure. For this purpose, we used stalled replication forks that were either isolated over a 2D-gel (Figure 9A) or just enriched by a CsCl-Actinomycin D gradient (Figures 9B and 9C). Furthermore, we analysed “early RFB molecules” (Figure 9B) and “late RFB molecules” (Figure 9C), because it is conceivable that Holliday-like DNA structures occurred after a time lag. In this case, only “late RFB molecules”, i.e. molecules that have already been stalled for a certain period of time, would adopt such a structure. We have inspected electron micrographs of 194 RFB molecules isolated over a 2D-gel and 152 molecules isolated by a CsCl/Actinomycin D gradient. Only one out of these 346 molecules could possibly be interpreted as a Holliday-like structure. This suggests that the annealed nascent strands of these structures, if they do exist, are either shorter than the resolution of the EM analysis (about 50 bp, (Portmann et al., 1976)) or that these structures are very transient and of such a low abundance that they are not detected in this assay. See also Figures 2a, 2b in (Lucchini and Sogo, 1994), and Figures 2d, 2e and 3a-c in (Lucchini and Sogo, 1995).
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Figure 9: EM analysis of psoralen crosslinked replication forks stalled at the RFB reveals no DNA structures indicative of Holliday-like intermediates. (A) Electron micrograph of a BglII, SphI digested replication fork that had been isolated over a 2D-gel. The branchpoint (arrowhead) of the stalled replication fork clearly consists of a three-way DNA junction. The putative 5' and 3' ends of the rRNA genes are indicated. Regions with single-stranded bubbles are indicative of nucleosome-covered, nontranscribed genes, whereas double-stranded regions are heavily crosslinked, which indicates that the rRNA gene is devoid of nucleosomes and transcriptionally active (Hanson et al., 1976; Conconi et al., 1984). (B) Electron micrograph of a replication fork stalled at the RFB (arrowhead) shortly after the event of stalling ("early RFB molecule"). The diverging replication fork, moving in the same direction as transcription, is still located on this DNA molecule (replication fork at the upper right). For this experiment, CsCl-enriched, psoralen crosslinked rDNA was digested with PvuII and subsequently analysed by EM (without prior 2D-gel isolation). Again, no DNA structure indicative of a Holliday-like intermediate can be observed (see also twofold enlargement of the branchpoint of the stalled replication fork). The bar represents 1kb. (C) Same experiment as in (B), but showing a replication fork stalled that had been stalled for a longer period of time (the divergent replication fork has moved off the DNA molecule, "late RFB-molecule"). As in (A) and (B), the branch point is a three way junction.
2.4.5 The stalled replication fork at prokaryotic termination sites

The best-characterised site-specific replication termination sites are the Ter-sites in *E. coli* and *B. subtilis*. They are dependent on a trans-acting factor, the Tus-protein and work, as the RFB in *S. cerevisiae*, in a polar fashion (for reviews see (Baker, 1995; Bussiere and Bastia, 1999; Hill, 1992)). However, contrary to our results of the stalled replication fork in *S. cerevisiae*, the leading strand has been shown to be arrested 63 to 65 bp ahead of the 5' end of the lagging strand at the Ter-site of *E. coli* (Hill and Marians, 1990; Mohanty *et al.*, 1998). Thus, a stretch of single-stranded DNA is exposed on the newly synthesised lagging arm. This might be a consequence of differences in the lagging strand processing at the stalled replication forks. Whereas, according to the model described above, the last Okazaki fragment of the eukaryotic lagging strand gets elongated after the stalling of the replication fork, such a mechanism might be absent in prokaryotes. In this respect, the prokaryotic replication fork arrested at the Ter-site would resemble more closely a replication fork in the process of elongation. It is conceivable that exposing such a long stretch of single-stranded DNA over a prolonged period of time would have a deleterious effect on *S. cerevisiae* by, for instance, providing a target for nuclease attacks. Thus, a special mechanism to prevent such a stretch of single stranded DNA at the RBF may have evolved in *S. cerevisiae*. In this context, it is noteworthy that the Okazaki fragments of prokaryotes are about a 1500 bp in length (Kornberg and Baker, 1992), which is longer than the eukaryotic ones that are about 40 to 300 bp in size (DePamphilis and Wassarman, 1980). Additionally, even though the general mechanism of DNA replication seems to be very similar in eukaryotes and prokaryotes, a closer look reveals considerable differences in the mechanisms of lagging strand synthesis and processing (Waga and Stillman, 1994). This difference may also be reflected in the mechanisms of replication fork stalling.
2.4.6 Digestion of RIs with endo VII

We have investigated the structure of RIs stalled at the RFB with the help of Endo VII, an enzyme specifically recognising branched DNA molecules. As expected, the RIs represented a good substrate for the Endo VII and were efficiently resolved into two linear DNA molecules. Moreover, it is the leading strand only, which was released. Endo VII has been shown to resolve a variety of branched DNA structures by introducing staggered nicks across the junction (reviewed in (Kemper, 1997)). These nicks are located two to six bp 3' of the branch point. Of the branched DNA structures investigated so far, "open Y-structured" DNA molecules (Jensch and Kemper, 1986) bear the closest resemblance to RIs. However, a major difference between these synthetically constructed DNA molecules and the naturally occurring RIs is the composition of the three arms. Whereas RIs contain two homologous arms, the synthetic Y-structures consisted of three arms with no homology to each other. Endo VII has been shown to introduce nicks in each of these non-homologous arms (Jensch and Kemper, 1986). In clear contrast, we have found that only the two homologous arms of the RIs were nicked by Endo VII, that is the parental leading and the nascent lagging strand (both 3' to the junction, see Figure 7C). This cleavage pattern can be explained by the symmetry of the enzymatic cleavage. Endo VII has been shown to bind the branched DNA and to execute its activity as a homodimer ((Birkenbihl and Kemper, 1998; Kosak and Kemper, 1990; Pohler et al., 1996)). Dimerisation is a prerequisite for the enzyme's activity (Pohler et al., 1996). Each dimer contains two active sites (Raaijmakers et al., 1999) and nicks both strands in independent but temporally closely related reactions within the lifetime of the enzyme-junction complex (Giraud-Panis and Lilley, 1997; Pottmeyer and Kemper, 1992). Due to the high symmetry of the homologous arms nicks are introduced preferentially into these two arms in the RIs. Holiday structures which consist of two pairs of homologous arms are resolved by cleavage of one of the two homologous pairs of arms (Kemper et al., 1984; Mizuuchi et al., 1982). Cleavage of one homologous and one non-homologous arm at the same time has never been observed (Kemper et al., 1990). By this way, the resolution of holiday structures and, as we have shown in the present work, of RIs into genetically sensible products is ensured.
2.4.7 Future directions

We have set up an assay that provides the means to analyse the nascent strands of RIs at nucleotide resolution by using a combination of 2D gel electrophoresis with primer extension or, alternatively, Southern analysis of a sequencing gel. In this work a population of Y-shaped DNA molecules containing an arrested replication fork was scrutinised. It would be interesting, however, to analyse different RIs from various stages of the replication process. For instance, RIs isolated from the Y-arc would contain a population of “dynamic”, i.e. progressing replication fork. The high-resolution analysis of the nascent strands could provide valuable information on the processing of the nascent lagging strand. However, since replication is a fast process, the abundance of such “dynamic” replication forks is very low, which could be a limitation to this assay. Another very interesting RIs that could be analysed this way would be X-shaped RIs intermediates where the forks are just about to merge. These RIs migrate in a discrete spot at the end of a spike emanating from the RFB. Preliminary experiments have shown that, despite their low abundance, such RIs containing terminating replication forks can be isolated (Figure 10). The analysis of the nascent leading and lagging strand of both replication forks on these RIs could allow drawing conclusions about the molecular events occurring when two replication forks fuse, thus termination the replication.

Finally, this assay could easily be extended to all sorts of branched DNA molecules that occur, for instance, in recombination or unusual DNA sequences, giving rise to secondary structures. Such structures can be treated with modifying enzymes recognizing branched DNA structures, such as endo VII. Subsequent analysis at high resolution could provide detailed information on the nature of such structures.
Figure 10: Purification of terminating RIs at the RFB. (A) Schematic representation of the isolated DNA molecules from a 2D gel: (1) terminating molecules containing two converging replication forks, (2) leftward-moving replication forks stalled at the RFB and (3) linear non-replicated molecules. (B) Southern analysis of isolated RIs from the indicated spot on the 2D gel.
2.5 Materials and Methods

2.5.1 Strains and culture conditions

*S. cerevisiae* A1 (MATα ade2-101 ura3-52 his3Δ200 lys2-801 Δbarl::LYS2) was used for all but one set of experiments, for which *S. cerevisiae* FTY 23 (MATα ura3-52 his3-1 gal2gal11trp1 URA3/YRp TRURAP) (Thoma, 1986) was employed to confirm the position of the arrested lagging strand at the RFB. Unsynchronised yeast cells, grown in complex medium (Sherman et al., 1986) at 30°C to a density of about 6*10⁶ cells/ml, were used as a source for rDNA isolation.

2.5.2 Primers

Only PAGE-purified primers were used for the primer extension reactions. All primers were diluted in water to the appropriate concentrations (1pmol/μl to 10pmol/μl). For a summary of all primers see appendix 7.2.

2.5.3 rDNA isolation and purification of RIs by preparative 2D gel electrophoresis

Chromosomal DNA of the early log-phase yeast cells was isolated by the glass-bead-method as described in (Huberman et al., 1987) and the rDNA was enriched on CsCl gradients essentially as described (Macleod and Bird, 1982). Briefly, to DNA isolated from about 10¹⁰ yeast cells, CsCl and Actinomycin D (Sigma, 2mg/ml stock solution) were added to a refractive index of 1.3848 and a concentration of 60μg/ml, respectively. The mixture was centrifuged at 4°C for at least 48 hours in a Beckman VTi 50 rotor at 26000 rpm for 65 hours. The gradient was fractionated from top into 1.2ml fractions using an ISCO fraction collector (model 640). To localise the rDNA, an aliquot of
each of these fractions was spotted onto a Biodyne B membrane (Pall) and hybridised with an rDNA-specific probe. The pooled fractions were desalted using a centricon-30 centrifugal concentrator (Millipore). No further enrichment of RIs with BND cellulose was performed, because a considerable amount of the replication forks stalled at the RFB elute with the linear DNA molecules (Linskens and Huberman, 1988). The rDNA was digested to completion with BglII (Axon Lab) and subjected to neutral/neutral 2D agarose gel electrophoresis as described by Brewer and Fangman (Brewer and Fangman, 1987), with some modifications. Per slot, about 10μg of the purified rDNA was loaded onto a 0.5% agarose gel (type II-EEO; Sigma) and run at 1V/cm for 16 hours in the first dimension. The second dimension was run in 1% low-gelling-temperature agarose (SeaPlaque/FMC) at 4V/cm for 8 hours. A gel slice was cut at the position of the apex of the Y-arc with the help of an autoradiogram of an identical 2D gel, which had been blotted and hybridised. Another gel slice containing the monomers was cut out under a long wavelength UV-lamp. The DNA was recovered by digesting the agarose with AgarACE (Promega) according to manufacturer’s protocol and subsequent ethanol precipitation with unspecific carrier DNA added.

2.5.4 Restriction enzyme, RNase H and endo VII digests

Restriction digests were carried out in the conditions recommended by the supplier. All restriction enzymes with the exception of SnaI, which was replaced by its isoschizomer Bst1077I (MBI Fermentas), were purchased from Axon Lab. RNase H (MBI Fermentas) digestion of RIs were carried out in a total volume of 50 μl of the supplied buffer (20mM Tris-HCl (pH 7.8), 40mM KCl, 8mM MgCl₂, 1mM DTT) at 37°C for 30 min. 4U of enzyme were employed to digest a total amount of 1μg RIs and carrier DNA. For endo VII digests, a total amount of 1μg DNA consisting of RIs and carrier DNA was incubated in 50 μl endo VII buffer (50 mM Tris pH 8, 10 mM MgCl₂,
50 mM NaCl, 1mM DTT, 100μg/ml BSA) for 30 min at 37°C, using the indicated amount of endo VII (a gift of B. Kemper).

2.5.5 Agarose gel electrophoresis and Southern transfer

Agarose gel electrophoresis was carried out in 1% agarose at 2V/cm without ethidium bromide. The gels were alkaline transferred onto a Biodyne B membrane (Pall) and was hybridised as described in (Lucchini and Sogo, 1992). As hybridisation probes, two PCR-amplified rDNA fragment (spanning nt 101 to 1088 and nt 101 to 366, respectively, see also Figure 3A) and the 4577 bp BgIII rDNA fragment encompassing the RFB, previously subcloned into a pUC18 vector, were utilised. The probes were labelled with [α-32P]dCTP (Amersham, 3000Ci/mmol) using the random prime oligo-labelling kit (Pharmacia) according to the manufacturer’s instructions.

2.5.6 Primer end-labelling

In a 25 μl reaction, 10 pmol of purchased (Microsynth) synthetic oligonucleotide was end-labeled with 10 pmol γ-32P ATP (Amersham, 3000 Ci/mmol) using 10 U T4 Polynucleotide kinase (Bio Labs). The sample was incubated at 37°C for 30 to 60 min. The γ-32P-ATP not incorporated was separated from the end-labeled oligonucleotide by a TE equilibrated Sephadex G50 column.

2.5.7 Primer extension

The sequences of the primers annealing to the top strand (parental leading and nascent lagging of the RIs stalled at the RFB) are as follows: primer 580, 5'-GGAACTTGCCATCATCATT-3' (nt 580 to 561); primer 483, 5'-CTCTTACATCTTTCTGGTA-3' (nt 483 to 464). The sequence for the primer anneal-
ing to the bottom strand (parental lagging strand of RIs): Primer 256, 5'-GATGGGTTGAAAGAGAAGG-3' (nt 256 to 274). All primers were PAGE purified and purchased from Microsynth. Primer end-labelling and the primer extension reaction were carried out as described previously (Wellinger and Thoma, 1996) with the following parameters: 30 amplification cycles consisting of 3 steps: 94°C for 45s, 55°C for 4 min, 72°C for 3 min. The DNA was precipitated by 0.1 volumes of 3M sodium acetate and 4 volumes of ethanol. The primer extension products were fractionated on 6% polyacrylamide gel containing 50% urea and TBE (Sambrook et al., 1989). Dideoxy Sequencing (Sanger et al., 1977) was carried out with CsCl purified rDNA as template using the same oligonucleotide as in the corresponding primer extension reactions.

2.5.8 Analysis of the nascent leading strand

2D gel purified RIs and monomers were digested to completion with Bst1077I (i-SnaI), the enzyme was removed with Strataclean (Stratagene) and the DNA was fractionated on a 6% sequencing gel containing 50% urea and 1xTBE. Immediately after the electrophoresis, two 15 x 20 cm pieces of wet Biodyne B (Pall) membrane, one for the upper and one for the lower part of the sequencing gel, were carefully placed onto the region to be transferred. A blotting stack was assembled by putting the gel and membrane between two, in 0.5 x TBE pre-equilibrated, extra thick blotting papers (Bio-Rad). The DNA was electro-botted in 0.5 x TBE in a Trans-Blot SD semi-dry electrophoretic transfer cell (Bio-Rad) at 40 mA for 30 minutes according to the instructions of the manufacturer. Subsequently, the DNA was cross-linked to the membrane by UV-exposure with a UV transilluminator (Bio-Rad). Finally, the membrane was sequentially hybridised with a strand-specific probe recognising the nascent leading strand (and the parental lagging strand) of the RIs and one recognising the nascent lagging strand (and the parental leading strand), respectively. The strand-specific probes were prepared as described in the protocol of Ruven et al. (Ruven et al., 1994): a PCR fragment spanning the region between the SnaI and HpaI restriction sites was generated by amplifying CsCl purified rDNA with primer 418, 5'-CAGGACATGCTTTGATATG-3' (nt 418
to 438) and primer 666, 5'-TACATGTATATATTGCACTGG-3' (nt 646 to 666). The primers were completely removed from the PCR product by two rounds of High Pure PCR Product Purification Kit (Roche Molecular Biochemicals). Subsequently, the purified PCR-product was utilised as template for primer extension in the presence of \([\alpha-^{32}\text{P}]d\text{CTP}\). 10 amplification cycles consisting of 3 steps were carried out; 94°C for 45 s, 56°C for 5 min, 72°C for 3 min. The primer extension product of primer 418 was used to detect the nascent leading strand, whereas the one of primer 666 to detect the nascent lagging strand. The strand specificity of the probes was checked by hybridisation to an rDNA restriction fragment with one end blunt and the other 5 bp overhanging, which had been fractionated on a sequencing gel. The sequencing lanes were generated by primer extension of 5'-endlabelled primer 256 as described above and were not hybridised. After the electroblotting the part of the membrane, where they were immobilised was cut off and directly exposed on an X-ray film.

2.5.9 EM analysis of replication forks stalled at the RFB

Psoralen crosslinked DNA, prepared exactly as described in (Lucchini and Sogo, 1994), was enriched over a CsCl-Actinomycin D gradient as described in 2.5.3. The DNA was either purified over a 2D-gel (see 2.5.3) or directly used for EM analysis, which was carried out essentially as described (Sogo et al., 1984). Briefly, psoralen crosslinked DNA was incubated for 60 min in a buffer containing 72% (v/v) formamide, 0.4M glyoxal, 2 mM Tris-HCl (pH=8.0) and 0.2mM EDTA. The denatured DNA was diluted with 30mM triethanolamine chloride (pH=7.8) to 50% formamide and was spread onto redistilled water by the BAC method as described in (Sogo et al., 1979).
3 IN VITRO RECONSTITUTION OF THE REPLICATION FORK BARRIER

3.1 Abstract

A replication fork barrier (RFB) at the 3’ end of yeast ribosomal genes blocks replication fork movement in a polar fashion. Sequences from the 3’ end of the yeast ribosomal RNA (rRNA) genes that are required for a functional RFB, were fused to an SV40-based vector. The constructs were replicated in vitro in the presence of SV40 T-antigen and a cytosolic extract prepared from human cells, and the progression of the replication forks was monitored by 2-dimensional gel electrophoresis. No pausing of the replication forks at the introduced yeast sequences was observed, suggesting that the yeast sequences as such do not impede the replication fork progression, and that no factors capable of complementing missing yeast protein(s) are present in the human cytosol. Supplementation with a crude whole-cell yeast extract completely abolishes in vitro replication. The extract was fractionated by chromatography, and the individual fractions were monitored by electromobility shift assays (EMSAs) for proteins binding to the DNA sequence required for the RFB. A DNA binding activity, specifically binding to this sequence, was identified and partially purified. No other DNA binding proteins binding in that region have been reported so far. In parallel, a protein that had been shown to be required for the RFB, Fob1p, was cloned and overexpressed in E. coli. Using EMSAs, a weak but specific binding of the DNA sequence required for the RFB could be assigned to the overexpressed Fob1p. Furthermore, in vitro replication of a plasmid with four tandemly repeated RFB sequences in the presence of purified Fob1p resulted in a slowing down of the replication fork at the repeated sequences. Thus, the Fob1p might be a DNA binding protein that, possibly with more protein(s), mediates the efficient replication fork arrest that was observed in vivo. Finally, a polyclonal α-Fob1 antibody was raised in rabbits. The antibody has been tested and characterised. Immunoprecipitation revealed that only minute amounts of Fob1p are present in the crude
whole-cell yeast extract. Thus, the DNA binding activity we have found in fractionated yeast extracts is probably distinct from Fob1p, a finding that is corroborated by SDS-PAGE analysis.

3.2 Introduction

There is indisputable evidence that replication and transcription take place concurrently in the rRNA gene cluster of *S. cerevisiae* (Saffer and Miller, 1986). However, such a simultaneous occurrence of the two processes inherently bears an antagonism, because both of them use the same template to direct their activity and because they move with considerably different velocities. In yeast, the replication fork travels about two times faster than the RNA polymerase (Alberts, 1994), whereas in *E. coli*, replication proceeds at 20 times the rate of transcription (French, 1992). Thus, collisions between the two processes seem to be inevitable. This conflict, however, seems to be less severe when replication forks and RNA polymerases move co-directionally. As direct consequence, the genome of most prokaryotes is organised in a way that replication forks move in the same direction as the RNA polymerases of heavily transcribed genes or large transcription units (operons) (Brewer, 1988; Sanderson and Roth, 1988; Zeigler and Dean, 1990). In the case of an actual collision, different outcomes seem to be possible. A bacteriophage T4 replication machinery assembled *in vitro*, for instance, can pass a ternary transcription complex of *E. coli* without displacing the nascent transcript, if both processes are co-directional (Liu *et al.*, 1993). If they move in opposite directions, the resulting head-on collision leads to a template switching of the bypassed RNA polymerase in that system (Liu and Alberts, 1995). In contrast, electron microscopic studies of rrnB, a ribosomal RNA operon of *E. coli*, indicate that the nascent transcripts are displaced when a replication fork invades the transcription unit from either direction (French, 1992). The movement of the replication forks, however, was hardly affected by co-directional transcription, but significantly retarded by transcription in the opposite direction.
Whereas all five rrn operons of *E. coli* are arranged co-directionally in respect to the replication fork movement (Brewer, 1988), a similar arrangement of the heavily transcribed rRNA gene cluster in *S. cerevisiae* is impossible, because bi-directional replication initiates manifold within the rRNA cluster (Linskens and Huberman, 1988; Fangman and Brewer, 1991). This means that one fork moves co-directional with transcription, whereas the other head-on towards the 3’ end of the rRNA genes. However, a specific mechanism seems to have evolved to protect rRNA transcription units from invading replication forks: the replication fork barrier (RFB) at the 3’ end of the rRNA transcription unit (Brewer, 1988; Linskens and Huberman, 1988; Brewer *et al.*, 1992). The RFB seems to be a very conserved feature in the mode of eukaryotic rDNA replication and has been found in many disparate eukaryotic organisms (Hernandez *et al.*, 1993; Little *et al.*, 1993; Wiesendanger *et al.*, 1994; Gencheva *et al.*, 1996; Sanchez *et al.*, 1998). Initially, it was hypothesised that transcription itself might be responsible for the stalling of the replication fork (Brewer and Fangman, 1988). However, there are three lines of evidence contradicting this hypothesis (Brewer *et al.*, 1992). First, an RFB has been observed even in the absence of ongoing polymerase I transcription. Second, the RFB persists when specific sequences from the vicinity of the 3’ end of the rRNA transcription unit are introduced into an episomal yeast plasmid. Finally, an rRNA transcription unit lacking these sequences, inserted into a yeast plasmid, does not generate an RFB. In this strain, however, the rRNA genes are transcribed by RNA polymerase II. Thus, the RFB does not seem to be a direct consequence of ongoing transcription. Instead, the most plausible explanation that has emerged so far is that the RFB is mediated by a trans-acting factor(s) that specifically binds to the RFB sequence (Brewer *et al.*, 1992; Kobayashi and Horiuchi, 1996).

It has been shown previously that transcriptionally active and inactive rRNA genes copies coexist in yeast throughout the cell cycle (Conconi *et al.*, 1989; Dammann *et al.*, 1993). Remarkably, the RFB has been found only in front of actively transcribed rRNA genes (Lucchini and Sogo, 1994) suggesting that the RFB activity and rRNA transcription are highly correlated. Even though, at first glance, this data seems to contradict the above mentioned evidence arguing against such a correlation, both findings can be reconciled by the presence of factors, independent of ongoing transcription,
which bind to the regulatory sites for transcription and/or the RFB activity. This notion is supported by the spatial arrangement of the regulatory sequences of polymerase I transcription termination and the sequences required for the RFB. They are located next to each other, both within a transcriptional enhancer at the 3' end of the rRNA transcription unit. Furthermore, the finding of nucleosome-free enhancers in the RNA polymerase I mutant strain (Dammann et al., 1995) strongly argues for this hypothesis.

No factors binding to the RFB sequence has been identified so far. However, two proteins, which bind to cognate sequences located within the enhancer sequences, upstream of the sequences required for the RFB, have been found, namely Reb1p and Abf1p (Morrow et al., 1989; Lang and Reeder, 1993). The Reb1p binds 15 bp downstream of the presumable bona fide polymerase I terminator of yeast and is an essential component of it (Lang and Reeder, 1993). The Abf1p (ARS binding factor) seems to act as a transcription factor participating in the regulation of many genes and, additionally, it seems to be involved in regulating the initiation of DNA replication (Toone et al., 1997). However, these two proteins, most likely, do not directly, or at least not without the help of additional factors, elicit the replication arrest at the RFB, since sequences encompassing their cognate binding site are not required for the RFB, nor do they increase the efficiency of RFB activity when transplanted onto an episomal plasmid containing the minimal sequences essential for the RFB.

Evidence for an involvement of the transcription termination factor (TTF-I) in the murine and human RFB was found by an in vitro replication approach (Gerber et al., 1997), using the SV40 based system (Li and Kelly, 1984). It seems that the murine and human RFB share TTF-I as trans-acting factor with transcription termination activity, because DNA binding TTF-I is required for both processes (Grummt et al., 1985; Gerber et al., 1997). TTF-I is a DNA binding protein that binds to an 18 bp sequence motif, pragmatically called Sal-box because of the presence of a SalI restriction site, that is repeated 10 times downstream of the 3' end of the rRNA gene. However, TTF-I does not seem to arrest the progressing replication fork single-handed, but rather as part of a larger protein complex, since additional DNA sequences with no cognate binding site for TTF-I are required for in vitro reconstitution of the RFB. How far the in vivo situation is reflected by this system remains to be elucidated because, several closely spaced
fork arrest site that co-localise with the transcription terminator elements have been found in vivo (Lopez et al., 1998). According to the in vitro results, the replication machinery ignores these sites and stops exclusively at the site closest to the 3’ end of the rRNA gene but one. This site coincides with the bona fide transcription termination site. It should be noted that the yeast equivalent of TTF-I, Reb1p, seems to be dispensable for the yeast RFB activity.

Genetic analysis of mutants defective in enhanced recombination activity attributed to sequences in the yeast NTS (for more thorough coverage of the connection between the RFB and recombination see introduction 1.2.2 and 2.2) led to the identification of the FOBI gene that is required for both, recombination and RFB activity (Kobayashi and Horiuchi, 1996). The Fob1p represents an attractive candidate for a trans-acting factor, which might bind to a cognate DNA sequence at the 3’ end of actively transcribed yeast rRNA genes and thus impede the progressing replication forks.
3.3 Aim and Scope of the Project

Our aim was to study how transcription and replication are co-ordinated in the ribosomal RNA genes of *S. Cerevisiae* during the cell cycle. For this purpose we intended to establish an SV40 based *in vitro* replication system that, supplied with a transcription competent whole-cell yeast extract, would have allowed concomitant transcription and replication of the same DNA or chromatin template. This way we hoped to reconstitute the yeast RFB *in vitro* eventually, which in turn would allow us to dissect the molecular mechanism of the RFB. The precise sequence requirement of RFB activity, for instance, could be scrutinised in such a system. Furthermore, regulatory proteins, possibly with a dual function in transcription termination as well as RFB activity might be identified by this system because the complexity and technical limitations of an *in vivo* assay could be circumvented.

When we started with the project, neither the FOB1 gene, nor the involvement of TTF-I in the RFB activity of mouse and human cells, had been identified. The discovery of FOB1, a yeast gene essential for the RFB, which was reported during the ongoing project, provided us at once with a trans-acting factor involved in the RFB activity. As a consequence, priority was given to the expression and characterisation of the Fob1p with the aim of reconstituting the RFB *in vitro* through the activity of Fob1p.
3.4 Results

3.4.1 The cis-element essential for the yeast RFB does not impede the progression of the replication fork in vitro

In order to dissect the molecular mechanism of the RFB and to identify putative trans-acting factors, a set of plasmids based on pJYM (Lusky and Botchan, 1981) was constructed (see materials and methods 3.6.3). All of these plasmids contain an SV40 origin of replication (ori) and support in vitro replication in the SV40 based replication system first described by Li and Kelly (Li and Kelly, 1984). Yeast sequences required for the blockage of the replication fork at the RFB were introduced into the plasmids in close proximity to the SV40 ori and in such an orientation that the replication fork emanating from the nearby ori would be impeded (anticlockwise moving replication fork, see Figure IIA, appendix 5.3). The rationale was that due to the close vicinity of the ori and the block (or pausing if the block was inefficient) of the replication fork, the majority of the plasmid would be replicated unidirectionally by the replication fork moving in clockwise direction. Such an asymmetric replication can be easily detected by neutral/neutral 2D gel electrophoresis, in particular by the accumulation of RIs at the blocking (pausing) site (Brewer and Fangman, 1988).

To this end, three plasmids containing sequences from the yeast rRNA transcriptional enhancer (Schultz et al., 1993) were constructed (see appendix 5.3). Plasmid pJYM-RFB carries the minimal sequence required for the RFB, the 
Hpal-HindIII fragment of the rRNA enhancer (Elion and Warner, 1984; Elion and Warner, 1986; Schultz et al., 1993), whereas pJYM-Enh accommodates the whole enhancer, which includes, besides the sequences required for the RFB, the sites where transcription termination takes place (Kulkens et al., 1992; Lang and Reeder, 1993). We reasoned that this plasmid might be more efficient in impeding the replication fork, since additional sequences towards the 3' end of the rRNA transcription unit have been shown to be required for full RFB activity (Brewer et al., 1992). Finally, pJYM-Term contains the part of the enhancer where transcription termination takes place, but lacks the sequences essential
for the RFB. Thus, in contrast to the afore-mentioned two plasmids, pJYM-Term does not contain sequences that elicit an impediment to the progressing replication fork.

In a first step, all three plasmid were replicated in vitro (Figure 11A). The resulting circular RIs were purified and digested with Clal and KpnI. The latter enzyme cuts just at the border of the SV40 ori. That way, two populations of Y-shaped RIs were generated and subsequently analysed by 2D gel electrophoresis. Figure 11B shows a representative 2D gel of the Y-shaped RIs of pJYM-Enh separated on a 2D gel. The 2D gel patterns of in vitro replicated pJYM-RFB and pJYM-Term looked virtually identical (data not shown). Two Y-arcs derived from the two restriction fragments as well as the triangle of the X-shaped, terminating molecules can be observed (termination occurs within a small region rather than a defined sequence). Clearly, no accumulated RIs are present on the Y-arc of the fragment encompassing the RFB (absence of prominent spot on the Y-arc, for details see chapter 2). In contrast, the descending part of the Y-arc towards the dimers (2n) shows a strong intensity, indicative of the replication fork having passed the RFB sequences and being close to entering the other restriction fragment. Consistently, signal for the X-shaped terminating RIs emanating from the bigger Y-arc is very intense, suggesting that a large fraction of the molecule is just about to terminate replication at roughly 180° from the ori. These data and the absence of accumulated RIs suggest that replication of pJYM-Enh is almost symmetrical (both replication forks move with similar velocity). Therefore, we conclude that the inserted RFB sequences as such do not impose any hindrance to the anticlockwise progressing replication fork. Furthermore, the human 293 cell extract is apparently not able to complement for yeast factors in order to reconstitute the yeast RFB.
Figure 11: The yeast RFB sequence as such does not impede the replication fork in vitro. (A) Schematic representation of the in vitro replication assay using pJYM-Enh as a template. pJYM-Enh consists of pBR322 sequences (depicted in black) to maintain the plasmid in E. coli as well as SV40 sequences (blue) including the SV40 ori enabling in vitro replication of the plasmid. The tRNA enhancer fragment of S. cerevisiae encompassing the sequences essential for the RFB (depicted in red) was subcloned into the plasmid in close proximity to the ori, in an orientation that anticlockwise replication.
forks would be impeded. Addition of T-antigen, S-100 293 cell cytosolic extract, NTPs, dNTPs (one of them α-32P-labelled) and Mg\(^{2+}\) with subsequent incubation at 37°C result in the plasmid's replication. By stopping the reaction at a time point at which replication is still going on, a population of circular RIs can be achieved (circular RIs). Upon purification and digestion with two restriction enzymes, one of them cutting at the ori, two populations of Y-shaped molecules will be generated, which were subsequently analysed by 2D gel electrophoresis. (B) 2D gel analysis of *in vitro* replicated pJYM-Enh digested with *KpnI* and *ClaI*. The upper panel shows a sketch illustrating the expected pattern of the resulting RIs. The two populations of Y-shaped molecules, derived from the two restriction fragments, result in two Y-arcs that ascend from the monomers (1n) of the corresponding fragment size (4877 bp for the longer fragment and 3698 bp for the shorter fragment, which encompasses the RFB) and return to the diagonal at the dimers (2n). The RIs of the larger fragment consist exclusively of rightward moving replication forks, whereas the ones of the smaller fragment of leftward moving replication forks. A part of the large fragment is entered by the leftward moving fork resulting in a population of X-shaped, terminating molecules (triangle at the upper part left of the big Y-arc). If the anticlockwise moving fork were impeded by the yeast RFB sequences an accumulation of RIs (prominent spot) would be observed at the position of the small Y-arc (indicated by an arrow) in the lower panel showing the result of an *in vitro* replication of pJYM-Enh. Clearly, no such accumulation of RIs can be detected.
3.4.2 Crude whole-cell yeast extract severely inhibits \textit{in vitro} replication

There are several good indications that the molecular mechanism of the blockage of replication fork at RFB occurs through a trans-acting yeast factor such as a DNA binding protein (see Introduction 3.2, (Brewer et al., 1992)). In agreement with this notion, we have found that the sequences required for the RFB \textit{per se} do not impede the progression of the replication fork \textit{in vitro} (Figure 11B). Therefore, in a next step, we intended to reproduce the pausing of the replication fork \textit{in vitro} by supplying yeast extract containing the necessary trans-acting factor(s) to the \textit{in vitro} replication reaction. Ultimately, such a system could be a useful tool to identify such a trans-acting factor(s).

For this purpose, we prepared a transcription competent whole-cell yeast extract (Schultz et al., 1991). We reasoned that this extract contained the required factors, because the RFB has been found only at the 3' end of transcriptionally active genes, suggesting an interplay between the processes of transcription and replication fork blockage at the RFB (see introduction 3.2, (Lucchini and Sogo, 1994)).

Two \textit{in vitro} replication strategies were employed: first, pJYM-Enh was preincubated with crude yeast whole-cell extract (1\,$\mu$g to 10\,$\mu$g), the components for the replication were added afterwards and the whole mixture was incubated at 37°C and analysed as previously described. Alternatively, the plasmid was preincubated with all replication components but dTTP, and 1\,$\mu$g to 10\,$\mu$g crude yeast extract was added afterwards. In this case replication initiation is allowed taking place, and the replication machinery starts synthesising until it meets the first dATP on the template. That way, we wanted to ensure that the initiation of replication was not inhibited by the addition of the crude yeast whole-cell extract. Inhibition of \textit{in vitro} replication by chromatin was successfully overcome by this method (Gruss et al., 1993). However, in both cases, the \textit{in vitro} replication was so severely inhibited that we could not observe any signal indicative of ongoing replication (data not shown). Therefore, we conclude that the transcription competent yeast whole-cell extracts contains factors that inhibit \textit{in vitro} replication so strongly that elongation of replication \textit{in vitro} is completely abolished.
3.4.3 Fractionated crude whole cell contains a factor(s) binding to the cis-element essential for the yeast RFB

In order to circumvent the problem of the inhibition of in vitro replication by crude yeast whole-cell extract, we partially purified the extract. In a first step, the crude yeast whole-cell extract was loaded on a Q-Sepharose column and subsequently eluted with a linear gradient from 0-1M NaCl. The vast majority of the bound proteins eluted between 0.25 and 0.5M NaCl, as monitored by absorption at 260nm. In order to obtain a higher resolution, all protein-containing fractions were pooled, reloaded on a Mono-Q FPLC column and re-eluted by a linear gradient of 0-1M NaCl. This time, the proteins eluted as three peaks at 0.25-0.4M, 0.4-0.55M and 0.55 to 0.65M, respectively, as monitored by absorption at 260nm (Figure 12).

To test these fractions for the presence of a factor involved in the replication fork blockage at the RFB, we carried out electromobility shift assays (EMSAs). The rationale behind this was the assumption that the replication fork arrest at the RFB was caused by a protein that binds to the DNA sequence essential for the RFB. If such a protein was present in the partially purified yeast extract, it should be detected by EMSAs. A 45 bp double-stranded oligonucleotide mapping next to the HpaI restriction site, which spans about a third of the HpaI-HindIII fragment required for the RFB (Figure 13A), was chosen as probe. The end-labelled oligonucleotide was incubated with individual Mono-Q fractions and subsequently run in a 4% non-denaturing polyacrylamide gel. Figure 13B shows the elution profile of the Mono-Q fractionation monitored by EMSA. The fractions induce several retarded bands migrating at various positions. At first glance, a minimum of four different band-shift activities can be distinguished: one peaks in fraction #19, another one in #27 and two overlapping activities in fraction #31 and #33, respectively. This implies that these fractions contain various proteins with an affinity to DNA. This is not an unexpected result, since already the partially purified yeast extract, which was loaded onto the Mono-Q column shows at least three retarded bands.

However, only a sequence-specific band-shift activity is likely to elicit the RFB activity. Therefore, we carried out homologous competition experiments by adding an
excess of unlabelled RFB-45 oligonucleotide. Only one of these band-shift activities, the slower migrating band present in the fractions #32 to #34 was competable by an excess of unlabelled RFB-45 oligonucleotide. Figure 13C shows such a competition experiment with fraction #32. Two retarded bands of about equal intensities are present in fraction #32. The lower of these bands (arrow) disappeared completely at a molar excess of 20x unlabelled RFB-45. In contrast, the upper band is not competed until an excess of 500x molar. This clearly indicates that the faster migrating retarded band is due to a sequence-specific DNA-protein complex, whereas the slower migrating complex binds the RFB-45 oligonucleotide in a much more unspecific manner.

In a next step, we wanted to further purify the sequence-specific band shift activity. To this end, fractions giving rise to the sequence-specific band shift activity were pooled and further purified by running through a Heparin column (see purification scheme, Figure 12). The elution of the Heparin column showed a distinct peak, which was assayed for band-shift activity (peak I in Figure 12). However, no sequence-specific activity was observed in the fractions of this peak (data not shown). The flow-through of the Heparin column was reloaded onto a Mono-Q column and the collected fractions were assayed for band-shift activity. Fractions #20 to #24 (peak II in Figure12) retarded the RFB-45 oligonucleotide. In order to test the sequence-specificity of these fractions, homologous as well as heterologous competition experiments were carried out. Whereas the retarded band completely disappeared at a 20x molar excess of unlabelled RFB-45 (homologous competition, lane 3 of Figure 13D), no significant change in the intensity of the retarded complex can be observed at that molar excess in heterologous competition experiments with unlabelled oligonucleotide containing the SP-1 or AP-1 consensus sequence, respectively (these oligomers were chosen fortuitously, lanes 6 to 15 in Figure 13D). This clearly shows that the retarded band results from a DNA-protein complex specifically binding the RFB-45 oligonucleotide.
Figure 12: Purification procedure used to partially purify crude yeast whole-cell extract. The red arrows indicate the presence of fractions that retard the RFB-45 oligonucleotide sequence specifically. In a first crude purification step, yeast whole-cell extract was loaded onto a Q-Sepharose column. Bound proteins were eluted with a linear NaCl gradient. All fractions with eluted proteins were pooled and re-purified over a Mono-Q FPLC column for better resolution. Fractions that were found to retard the RFB-45 oligonucleotide specifically were pooled and reloaded on a Heparin column that was subsequently eluted with KPO4. The fractions eluted in a single sharp peak, which was termed peak I. The flow-through of the Heparin column was collected and repurified over another Mono-Q column. The eluted proteins were termed peak II in analogy to elution of the Heparin column. Peak II contained some fractions in the highest obtained purity that specifically retard RFB-45.
Figure 13: EMSA analysis of fractionated yeast whole-cell extract. (A) Schematic drawing illustrating the location of oligonucleotide RFB-45 in the context of the replication fork stalled at the RFB. (B) Elution profile of the Mono-Q fractionation of partially purified (see materials and methods for details) yeast whole-cell extracts. The number
above each lane corresponds to the fraction number collected by elution with a linear NaCl gradient. Fraction #15 eluted at about 0.2M NaCl, fraction #39 at 0.7M NaCl. Q indicates the pooled fractions of the Q-Sepharose column, which was the loading of the Mono-Q column. F points to the position of the free probe. (C) Homologous competition experiment of end-labelled RFB-45 incubated with Mono-Q fraction #30 in presence of increasing amounts of unlabelled RFB-45. Lane 1: no unlabelled RFB-45, lane 2: 20x molar excess of unlabelled RFB-45 over end-labelled RFB-45, lane 3: 50x molar excess, lane 4: 100x molar excess, lane 5: 250x molar excess, lane 6: 500x molar excess. (D) Homologous (lanes 1-5) and heterologous (lanes 6-15) competition experiment: end-labelled RFB-45 was incubated with fraction #22 of peak II (see Figure 12) and increasing amounts of homologous competitor DNA (unlabelled RFB-45) or heterologous competitor DNA (unlabelled SP-1 oligonucleotide, lanes 6-10 and unlabelled AP-1, lanes 11-15). The molar excesses of unlabelled DNA are from left to right: 0x, 5x, 20x, 100x and 500x.
We conclude from these data, that we have enriched a factor(s) specifically binding to the DNA sequence required for the RFB. Such a factor is likely to be involved in the blockage of the replication fork at the RFB. To our knowledge, until now, no DNA-binding protein has been reported to bind to a sequence within the region.

The partially purified fractions that specifically bind to the RFB-45 sequence were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). We hoped to identify candidate proteins that could be involved in the replication fork blockage at the RFB by scrutinising the fractions for proteins appearing in all active fractions. Unfortunately, there were still far too many proteins present in the partially purified fractions to allow drawing any conclusions in that respect (Figure 14A lane 1 to 5, see later and data not shown).

The fractions with specific binding activity were also added to in vitro replication experiments. However, even though these fractions inhibited the in vitro replication reaction much less, we were not able to detect the characteristic accumulation of RIs due to the RFB (see later).

### 3.4.4 Overexpression of Fob1p, a protein required for the yeast RFB, in *E. coli*

The identification of a protein required for the yeast RFB by Kobayashi and Horiuchi (Kobayashi and Horiuchi, 1996) provided us with a trans-acting factor mediating the replication fork arrest at the RFB. Since it proved rather difficult to further purify the specific band-shift activity we have detected in the partially purified yeast extract, further characterisation of this activity was severely hampered. Therefore we concentrated our efforts for the in vitro reconstitution of the yeast RFB on this novel protein, which had been termed Fob1p for “DNA replication fork blocking protein”.

Closer inspection of the FOB1 gene revealed that it is identical with the open reading frame YDR110w of the *S. cerevisiae* genome database (ID L0003959), and that it is located on chromosome IV (the rDNA repeats are located on chromosome XII). The protein is predicted to consist of 566 amino acids (Figure 15) with a molecular
weight of 65 kD and a pI=5.99. Hydropathy analysis (Kyte and Doolittle, 1982) reveals a stretch of hydrophobic amino acids (amino acids 267-283) and analysis of the charge distribution (Brendel et al., 1992) a cluster of positively charged amino acids (amino acids 345-365). Interestingly, Foblp exhibits no homology to any other protein listed in the data banks. Therefore, no information about its three dimensional structure is available at present. Furthermore, it contains a nuclear localisation signal (KRKP, amino acids 362-365) and has recently been shown to be a nucleolar protein (Defossez et al., 1999).

Since the FOB1 gene lacks introns, we cloned its cDNA directly by PCR using a high fidelity thermostable DNA polymerase. Subsequently, the PCR fragment was subcloned into the polylinker of a pGEX-KG vector (see materials and methods 3.6.3). pGEX plasmids are designed for high-level intracellular expression of genes as fusion with Glutathione-S-Transferase (GST) (Smith and Johnson, 1988). They allow inducible protein expression driven by an isopropyl β-D-thiogalactoside (IPTG) inducible tac promoter. A major advantage of this system is its convenient one-step purification procedure with glutathione sepharose.

The FOB1 gene was cloned in frame to the 3’ end of the GST-gene of pGEX-KG plasmid giving rise to the pGEX-Fobl. Expression of this plasmid in E. coli resulted in a fusion-protein of Foblp with an N-terminal GST as purification tag. The GST can be removed from the fusion protein by thrombin cleavage. Figure 14B shows an SDS-PAGE loaded with Foblp expressed in E. coli after the one-step purification with glutathione Sepharose. The GST-fusion protein has an apparent molecular weight of about 110kD (Figure 14B, lane 1), which is higher than the calculated molecular weight of 92 kD. Thrombin cleavage removed the GST efficiently from the fusion protein, thereby releasing a product with an apparent molecular weight of about 80 kD (Figure 14B, lane 2). The calculated molecular weight of the Foblp is 65 kD. However, it is not unusual for a protein to migrate on an SDS-PAGE at an apparent molecular weight, which differs from the calculated one. We conclude from the fact that the expression of pGEX-Foblp resulted in a thrombin cleavable product with an apparent molecular weight of 80kD that this product corresponds to the Foblp. If a frame-shift mutation had occurred, a truncated shorter product would have been generated. The GST-
Fob1 fusion protein was expressed in the cytosol of *E. coli* suggesting that it is soluble despite of its predicted transmembrane domain. However, the Fob1p expression required low temperatures and the yield was very poor. The fact that no detectable protein induction was observed upon IPTG addition in suggests that the low yield of GST-Fob1p is due to poor expression and not to aggregation leading to the loss of the protein in the insoluble fraction.

We have found a specific band-shift activity in partially purified yeast extracts. In order to determine whether these fractions contain partially purified Fob1p, we separated some of these fractions with the specific band-shift activity on an SDS-PAGE with Fob1p run in parallel (Figure 14A). Still too many proteins were present in fractions, which had been purified over less than three purification steps, to allow drawing any conclusions (Figure 14A, lanes 2 and 3). In the band-shift active fraction #22 of peak II (see Figure 12 for purification scheme) no band migrates at the position where the recombinant Fob1p migrates (Figure 14A, lane 4). The same is true for fraction #36 of peak I (no band-shift activity, Figure 14A, lane 5). Thus, the Fob1p was presumably not present in the pooled fractions that were loaded onto the Heparin column. However, it is possible that the Fob1p expressed in yeast runs differently on a SDS-PAGE, due to a post-translational modification such as a phosphorylation(s). Alternatively, if only minute amounts of the Fob1p are present in the partially purified yeast extracts, detection on a SDS-PAGE, even when silver-stained, would be difficult (see also 3.4.3).
Figure 14: Overexpression of Fob1p as a GST-fusion protein in *E. coli*. (A) SDS-PAGE analysis of partially purified yeast extracts in comparison to Fob1p overexpressed in *E. coli*. See Figure 12 for purification procedure. Crude yeast extract (lane 1), pool of Q-Sepharose fraction (lane 2), pool of Mono-Q fractions (RFB-45 specific band-shift activity, lane 3), fraction #22 of peak II (band-shift active, lane 4), fraction #36 of peak I (no band-shift activity, lane 5) and purified Fob1p overexpressed in *E. coli* (lane 6) were separated on a 15% SDS-PAGE and visualised by silver staining. (B) Purified recombinant proteins were separated on 10% SDS-PAGE and stained with Coomassie-Blue. Lane 1 shows the migration of the GST-Fob1 fusion protein, lane 2 of the Fob1p after Thrombin cleavage. M: Molecular weight marker.
Figure 15: Predicted amino acid sequence of Fobl. A putative trans-membrane domain spans amino acids 267-283 (shaded in blue), and a cluster of positively charged spans amino acids 345-365 (shaded in green) and is overlapped by the nucleus targeting KRKP motif (box).
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3.4.5 Fob1p binds to the yeast RFB sequence

Genetic evidence showing that Fob1p is required for the arrest of the replication fork at the RFB (Kobayashi and Horiuchi, 1996) makes Fob1p a good candidate for a DNA-binding protein, because, in analogy to the bacterial system, the replication fork could be impeded by such a DNA binding protein. If Fob1p acts through DNA binding, it is expected to retard an appropriately chosen oligonucleotide from the RFB region in an EMSA. Therefore, we carried out EMSAs with recombinant Fob1p and oligonucleotides spanning the region essential for the RFB. First, end-labelled RFB-45 oligonucleotide was incubated with Fob1p and analysed by non-denaturing PAGE (Figure 16A). We have found that RFB-45 is retarded by some fractions of the partially purified yeast extract (see 3.4.3). Likewise, recombinant Fob1p gives rise to a retarded band (Figure 16A, lane 2). This band disappears upon incubation with 20x molar excess of unlabelled RFB-45, whereas the same molar excess of unlabelled AP-1 oligonucleotide shows no effect on the retarded band (Figure 16A, lanes 3 and 4, respectively). This suggests that the retarded band is due to a sequence-specific Fob1p-DNA complex. However, only a minor fraction (less than 5%) of the RFB-45 oligonucleotide is bound by Fob1p and this ratio, which is in favour of the free oligonucleotide, could not be substantially changed by adding increased amounts of Fob1p. Thus, even though the Fob1p binds to RFB-45 in a sequence specific manner, the binding seems to be inefficient. The position of the retarded band induced by Fob1p was compared with the bands induced be adding of pooled Q-Sepharose fractions (Figure 16A, compare lanes 1 and 2). The retarded band migrates substantially faster than the bands of the Q-Sepharose fractions, thus the migration behaviour of the band retarded by Fob1p is different from the one of the retarded, sequence-specific band we have found in partially purified yeast extracts (co-migrating with the lower band of the Q-Sepharose fractions, see Figure 13C). This suggests that the DNA-binding activity of the extract and Fob1p are not identical.
In a next step, we wished to investigate whether Fob1p binds only to a sequence of the RFB-45 oligonucleotide (which is close to the major arrest of the nascent strands of the replication fork stalled at the RFB, see Figure 13A) or whether other regions of the 129bp HindIII-Hpal fragment essential for the RFB also represent a target for Fob1p binding. For this purpose, two oligonucleotides were chosen in a way that, together with the RFB-45, the whole HindIII-Hpal fragment was covered by the three overlapping oligonucleotides (Figure 16B, upper panel). All three oligonucleotides were incubated with Fob1p and subjected to an EMSA (Figure 16C, left panel). The slowest migrating band is due to the Fob1p (indicated by a bracket in Figure 16C). The other, faster migrating bands are due to co-purified *E. coli* proteins or degraded Fob1p (they only appear in preparations of lower quality, compare lane 2 of Figure 16A with lane 1 of Figure 16C and see discussion 3.5.3). Besides oligonucleotide RFB-45 also RFB-48, but not RFB-50, are retarded by Fob1p. This suggests that there is more than one binding site for Fob1p present in the HindIII-Hpal fragment. It has been speculated that the presence of the minor replication arrest sites at the RFB, which is located at the border of the RFB-48 oligonucleotide could be caused by a second binding site for the DNA binding protein (Brewer et al., 1992). Our finding supports this hypothesis.

Two potential consensus sequences for a putative DNA binding protein mediating the RFB have been reported (Hernandez et al., 1993; Ward et al.). Both are located within the RFB-45 oligonucleotide. In an attempt to further delineate the sequence requirement for the DNA binding of Fob1p, two mutated oligonucleotides, where these putative consensus sequences were deleted (Figure 16B, lower panel), were used for EMSAs. The band-shift activity of Fob1p was not abrogated by neither of the mutations suggesting that these consensus sequences are not required for Fob1p binding. However, the slower migration of the retarded band of the mutated oligonucleotides could be indicative of a conformational difference between the RFB-45 oligonucleotide and the two shorter ones.

The sequence-specific band-shift activity of the partially purified yeast extracts was not affected by deletion of these putative consensus sequences either (data not shown).
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Figure 16: Fob1p specifically binds to the sequence required for the RFB. (A) EMSA analysis of recombinant Fob1p overexpressed in E. coli. As a probe, end-labelled RFB-
45 was used. Lane 1 shows the gel shift of partially purified yeast extracts (pooled fractions of Q-sepharose), in lane 2-4 with recombinant 100ng of Fob1p. A 10x molar excess of unlabelled RFB-45 oligonucleotide (homologous competition) was added in lane 3 and a 10x molar excess of AP-1 oligonucleotide (heterologous competition) in lane 4. The position of the putative DNA-Fob1p complex is indicated by an arrow. F: Free probe. (B) Schematic drawing illustrating the various probes used for EMSAs with Fob1p. The upper panel shows the three overlapping probes spanning the whole RFB region. The lower panel shows the two mutated RFB-45 probes. RFB-38mt1 lacks the putative consensus sequence of Ward et al. (Ward et al.), indicated by B; RFB-38mt2 the one of Hernandez et al. (Hernandez et al., 1993), indicated by H. (C) EMSA analysis of Fob1p with the three probes spanning the whole region required for the RFB (lanes 1 to 3) and the two mutated RFB probes (lanes 5 and 6). The migration of the retarded bands due to a Fob1-DNA complex is indicated by brackets, all other bands are due to co-purified contaminants.
3.4.6 Analysis of the Fob1p-DNA protein complex by electron microscopy

To corroborate the evidence that Fob1p specifically binds to the yeast RFB sequence, an electron microscopic (EM) analysis of pSVori-Enh in the presence of purified, recombinant Fob1p was carried out. pSVori-Enh carries the yeast rDNA enhancer encompassing the sequences essential for the RFB. pSVori-Enh was cut with NcoI, which linearises pSVori-Enh. The enhancer region is located close to one end of the linearised molecule. Fob1p was then incubated with the linearised plasmid under the same conditions as used for the EMSAs. Protein-DNA complexes were crosslinked with glutaraldehyde, spread by the mica absorption method (Sogo et al., 1987) and subsequently analysed by electron microscopy. Protein-DNA complexes were observed in only about 1% of the molecules. A representative example of such a complex is shown in Figure 17A. Statistical analysis of these complexes showed that the vast majority of them are located close to the end of the DNA molecule, at a position of 590±98 bp (Figure 17B). In the NcoI linearised pSVori-Enh molecules, the sequences required for the RFB span position 542 to 672, thus overlapping the position of the DNA-protein complex. We conclude that Fob1p specifically binds the RFB sequence. However, the fact that only 1% of the DNA molecules were found to be complexed with a protein indicates that Fob1p binding is rather inefficient. Consistent with this observation, we have found only a small fraction (less than 5%) of the oligonucleotides being retarded by the Fob1p in the EMSAs (Figure 16).
Figure 17: EM analysis of Fob1 binding to the RFB sequence. (A) Representative electron micrograph showing a Fob1p molecule bound to the RFB sequence of pSVori-Enh. (B) Statistical analysis of the protein-DNA complex showing the binding site of Fob1p. The position of the sequence essential for the RFB on the linearised pSVori-Enh is shown in the lower part of the figure.
3.4.7 *In vitro* reconstitution of the RFB using Fob1p expressed in *E. coli*

As a next step, we wished to investigate if Fob1p, bound to the RFB sequence, is able to impede the progression of the replication fork *in vitro*. Since the binding of the recombinant Fob1p to the RFB sequence seems to be rather inefficient (1% in the EM analysis, less than 5% in the EMSAs), we constructed a plasmid containing four *Hind*III-*Hpa*I fragments, encompassing the sequences essential for the RFB in tandem (pJYM-RFB4, see materials and methods 3.6.3). All of them were introduced in such an orientation that the anticlockwise fork, emanating from the nearby SV40 ori, will be impeded (Figure 18A). We expected that the fraction of templates bound by Fob1p was substantially increased in this plasmid. If the Fob1p, bound to these sequences, causes the replication fork to pause or to arrest, an accumulation of RIs will be observed by a 2D gel analysis of purified, *Cla*I and *Kpn*I digested RIs. However, since pJYM-RFB4 contains at least four Fob1p binding sites, distributed over a relatively broad region (about 500 bp), the signal of the accumulated RIs is expected to give rise to a bulge rather than a spot on the otherwise smooth Y-arc (see Figure 18A).

pJYM-RFB4, pre-incubated with purified, bacterially expressed Fob1p or mock treated, was replicated *in vitro* in the presence of [α-32P]-dCTP, and subsequently purified and digested with *Kpn*I and *Cla*I. 2D gel analysis revealed that, in contrast to crude yeast whole-cell extract, pre-incubation with purified Fob1p did not abolish *in vitro* replication. However, the efficiency of the reaction dropped by a factor of about five (Figure 18B, the panel showing the reaction with Fob1p on the right was exposed about 5 times longer than the one on the left side).

The two restriction fragments give rise to two Y-arcs, one of them consists of a strong ascending and a weak descending part (Y-arc of the small restriction fragment), whereas the Y-arc of the bigger restriction fragment consists only of the ascending part of the arc and misses the descending one. This pattern is indicative of RIs in a relatively early stage of replication. The absence of the descending part of the Y-arc from the larger fragment can be explained by the asymmetric cleavage of the restriction enzyme. Before the clockwise-moving replication is able to reach the end of the restriction frag-
ment the anti-clockwise moving fork enters the fragment, thus giving rise to X-shaped Rls. Therefore, no Y-shaped DNA fragment with two long replicated arms and a short unreplicated arm are generated. Note that the *in vitro* replication of pJYM-Enh resulted in a similar pattern (see also Figure 11B: the ascending part of the slower migrating Y-arc displays a strong intensity, whereas the intensity of the descending part of the Y-arc is very weak). Only if the anticlockwise-moving replication fork was completely blocked at the introduced RFB sites, a full descending arc would be expected (if the template does not consist of multimeric plasmids). In this case, termination of replication would take place when the converging clockwise-moving fork met the stalled replication fork. Thus, the complete absence of the descending part of the slower migrating Y-arc indicates that the anti-clockwise moving fork is not permanently blocked at the tandemly repeated RFB sequences, neither in the mock treated reaction nor in the presence of Foblp. This and the absence of accumulated Rls suggest that four tandemly repeated RFB sequences do not impose an impediment on the progressing replication fork *in vitro*, indicating the no sequence-inherent structures are responsible for the RFB, even when these sequences are tandemly repeated.

A closer inspection of the fast migrating Y-arc of the DNA molecules replicated in presence of Foblp reveals a weak, but clearly visible, bulge-shaped region of higher intensity as compared to the corresponding Y-arc of the mock treated reaction. The position of this region coincides with the introduced RFB sequences. Furthermore, the intensity of the region around the inflection point of the Y-arc (where the ascendent and descendent sections of the Y-arc converge), as well as the descending part of it, are less intense when the reaction was incubated with Foblp, whereas the ascending arc shows a similar or even higher intensity. This indicates that in the presence of Foblp, the replication fork passes the small restriction fragment more slowly than the bigger one. Taken together, these data suggests that the anti-clockwise moving replication fork pauses, or at least moves substantially slower through the introduced yeast RFB sequences in the presence of Foblp. Considering the results showing that Foblp is able to bind to the RFB sequence, as well as the position and the shape of the accumulated Rls, we suggest that the observed pausing is due to Foblp bound to the yeast RFB sequence.
We have found that in some fractions of partially purified yeast whole-cell extract a factor that specifically binds to the RFB sequence was present. In order to find out whether this factor was able to pause the replication fork in vitro, analogous experiments as described above were carried out, but instead of Fob1p we pre-incubated the in vitro replication reaction with fraction #32 (band-shift active fraction obtained after first Mono-Q column, see Figure 12). Figure 18C shows a 2D gel of in vitro replicated pJYM-RFB4, mock treated (left panel) or pre-incubated with fraction #32. The efficiency of the in vitro replication reaction dropped by a factor of two to three upon addition of fraction #32. However, no accumulated RIs were observed at the expected position on the faster migrating Y-arc. This indicates that the factor binding to the RFB sequence, present in this fraction, is not able to pause a progressing replication fork and is therefore not identical with Fob1p. Furthermore, DNA binding alone does not seem to be sufficient for the pausing of the replication fork.
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(A) 4xRFB (yeast) KpnI In vitro replication -factor +factor

SV40 ori SV40
pJYM-RFB4

Clal pBR322

2D gel analysis

1st dim 2nd dim
- Factor + Factor

(B) control +Foblp
Figure 18: In vitro reconstitution of the RFB by purified Fob1p expressed in E. coli. (A) Schematic illustration of in vitro replication in presence of a trans-acting factor impeding the replication fork at the RFB (indicated with “factor”). pJYM-RFB4, which contains four tandemly repeated HindIII-HpaI fragments (encompassing the sequence essential for the RFB, depicted in red) in close proximity to the SV40 ori (sequences derived from SV40 are depicted in blue), is replicated in vitro. Both replication forks are assumed to progress at comparable velocities. If the antclockwise moving fork is impeded by a protein bound to the RFB sequence, an accumulation of a distinctive, Y-shaped DNA molecule (depicted in red) will be generated upon Clal-KpnI double-digestion. This accumulation can be detected by 2D gel electrophoresis. A bulge rather than a spot is will appear on a 2D gel, if the impediment of the replication fork occurs over a broader region and not at one defined position (arrow in sketch of 2D gel). (B) 2D gel analysis of in vitro replicated pJYM-RFB4 preincubated with recombinant Fob1p (right panel) or mock treated (left panel). The 2D gel on the right was exposed about five times as long as the one on the left. The arrow in the right panel points to the accumulated RIs detected as a bulge on the smooth Y-arc. (C) Similar experiment as in (B) with partially purified yeast extract, fraction #32 after first Mono-Q column (giving rise to a specific band-shift activity, see Figure 12) instead of recombinant Fob1p (right panel). The panel on the left shows the mock treated reaction.
3.4.8 Characterisation of a polyclonal α-Fob1 antibody

The availability of bacterially expressed, highly purified Fob1p gave us the opportunity to generate a polyclonal α-Fob1 antibody. To avoid potentially interfering proteins co-purified from *E. coli*, recombinant Fob1p was re-purified over an SDS-PAGE. Two rabbits, termed rabbit #5 and #6, respectively, were immunised with Fob1p and after administering two more immunisations to boost the production of antibodies, blood was collected and antiserum prepared. In a first step, the sera were tested for specificity and cross-reactivity. For that purpose, bacterially expressed Fob1p was analysed on a Western blot, incubated either with pre-immune or α-Fob1 antiserum of the two rabbits (Figure 19A). The α-Fob1 antisera gave rise to a major band that is not present in the pre-immune sera, indicating that the immunisation worked. The lanes incubated with pre-immune sera are either blank (rabbit #6) or give rise to a minor band (rabbit #5, empty arrow), which is also present in the lanes incubated with α-Fob1 antiserum. This band is due to a cross-hybridisation of the antiserum with a bacterial protein, co-purified with Fob1p.

There are at least two more bands (arrowheads in Figure 19A) present in the lanes incubated with antiserum, one of them almost co-migrating with the Fob1p. These bands could either be due to Fob1p-degradation products or, alternatively, due to co-purifying bacterial proteins. In order to determine the origin of these bands, three preparations of protein overexpression in *E. coli* were analysed by Western blot (Figure 19B). In two of them, the pGEX-Fob1 vector was overexpressed in *E. coli*, and subsequently eluted either by cleavage with Thrombin or by glutathione, yielding either Fob1p or GST-Fob1p, respectively (lane 1 and 2). The third was a preparation of empty pGEX-KG vector overexpressed in *E. coli*, yielding GST (data not shown). The band that migrates only slightly faster than the Fob1p appears in all three protein preparations implying that it is due to a co-purified protein from *E. coli*. Its migration close to the Fob1p suggests that this protein co-purified with the Fob1p during the purification of the Fob1p over an SDS-PAGE. Thus, the rabbits were most probably co-immunised with this protein and the detected band is not due to a degradation product of Fob1p nor to a crosshybridisation, but to a polyclonal antibody directed against this *E. coli* protein.
However, since Fob1p is a yeast protein, we do not expect that this *E. coli*-antibody would interfere with subsequent analyses.

In a next step, we wanted to know if the Fob1p was also present in the crude yeast whole-cell extracts we used for partial purification. However, no signal indicative of the presence of Fob1p in the whole-cell extract was detected by Western blot (Figure 19B, lane 4). Additionally, partially purified yeast extract, retarding the RFB-45 oligonucleotide in a sequence-specific manner, did not give rise to a signal either (data not shown). Only by increasing the sensitivity with the help of immunoprecipitation did we succeed in detecting Fob1p in the crude yeast whole-cell extract (Figure 19C, lane 5). This indicates that either the abundance of Fob1p in yeast is very low, or, more likely, that the Fob1p was lost during the preparation of the yeast extracts. Furthermore, this corroborates the notion that the activity present in the partially purified yeast extract leading to specific binding of a sequence within the region required for the RFB, is distinct from the Fob1p.
Figure 19: Characterisation of a polyclonal α-Fobl antibody. (A) Western blot of recombinant Foblp used for immunisation of two rabbits (rabbit #5 and #6). The lanes indicated with P were incubated with pre-immune serum, whereas α indicates incubation with the antiserum of the corresponding rabbit. The dilution factor of the antisera was 1:1000. The upper arrow indicates the migration of Foblp, the lower a crosshybridizing band. (B) Western blot of various protein preparations with α-Fobl antiserum of rabbit #6 (dilution 1:1000). Lane 1 shows the GST-Foblp fusion protein, lane 2 shows a preparation of bacterially expressed Foblp after Thrombin cleavage and lane 3 shows a preparation of GST (expressed from the empty pGEX vector). In lane 4 10 μg of crude whole-cell yeast extract were loaded. The arrow marked with a star points to the signal of a protein derived from E. coli. (C) Immunoprecipitation of bacterially expressed...
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Fob1p and of crude whole-cell yeast extract with the polyclonal rabbit α-Fob1 antibody. Lanes 1 to 3: Western analysis of yeast extract, Fob1p and GST-Fob1p, respectively. Lane 4 shows the immunoprecipitation of yeast extract with preimmune serum from rabbit #6, lane 5 with antiserum from the same rabbit. The arrow indicates the position of the IgG heavy chain.
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3.5 Discussion

3.5.1 In vitro replication of the yeast RFB sequences in the absence of yeast factors

In an attempt to reproduce the yeast RFB in a cell free system, yeast DNA sequences required for RFB activity in vivo were fused to SV40 origin-based plasmids. In vitro replication of these plasmids using T-antigen and a cytosolic 293 cell extract showed that the yeast DNA sequences as such did not impede an approaching replication fork. This result is in agreement with earlier findings, where the same sequences, replicated in E. coli, did not impede the replication fork movement (Brewer et al., 1992). Thus, our result corroborates the notion that a trans-acting yeast-specific factor(s) is required for the RFB activity.

Since the proteins of the replication machinery are supplied by a cytosolic extract of human 293 cells in the in vitro system, and since an RFB has also been found at the 3' end of the human rRNA genes (Little et al., 1993), we speculated that a homologous factor in the human cell extract, which was not present in E. coli, could complement for a yeast factor(s) mediating the RFB. Moreover, it has been reported that an analogous cytosolic extract from HeLa cells that supports in vitro replication was able to reproduce the RFB, when 3' terminal murine rDNA was fused to an SV40 ori based vector (Gerber et al., 1997).

The fact that we did not observe any stalling or slowing down of the progressing replication fork could indicate that the human and yeast factor are diverged too far to complement each other. This could be reflected in the finding that, in contrast to the polar block in yeast, the human RFB seems to work in both directions (Little et al., 1993). If the factor in question is a DNA binding protein, which seems to be a plausible assumption, the cognate sequence for the human protein might be missing in the yeast sequence required for the RFB and thus no block is observed in the in vitro replication, despite of the presence of the necessary factor(s). As a matter of fact, TTF-1 seems to be required for the murine RFB (Gerber et al., 1997). TTF-1 is known to bind a specific
DNA sequence termed Sal-box (Grummt et al., 1986; Bartsch et al., 1988), which does not occur in the yeast sequences required for the RFB.

Alternatively, the abundance of an RFB-inducing human factor could be too low in the cytosolic 293 extracts used for the in vitro replication, since such a factor may be located primarily in the nucleus. Additionally, the in vitro replication assay is not sensitive enough to detect an impediment of the replication fork if only a minor fraction (less than 10 percent) of the replicating molecules contain an RFB.

3.5.2 The search of a potential trans-acting yeast factor mediating the replication fork arrest at the RFB

Since we did not observe an impediment of the progressing replication fork through the yeast sequences required for the RFB by in vitro replication in cytosolic 293 cell extract, we sought to identify essential factor(s) mediating replication fork arrest by using a crude, transcription competent whole-cell protein extract from yeast. In a first attempt, we added unpurified crude whole-cell extract to the in vitro replication reaction. We reasoned that such a crude extract was more likely to contain all the required factors for the RFB than a partially purified extract, because the purification procedures might lead to the loss of such a factor(s). However, the complete abolishment of the in vitro replication reaction by the crude extract forced us to further purify the crude yeast extract.

The reasons for this drastic inhibition of the in vitro replication are most likely factors in the extract, such as nucleic acids and proteins that both, unspecifically and specifically inhibit the in vitro replication reaction. It has been found previously that the addition of a nuclear extract or the assembly of the DNA templates for in vitro replication into chromatin inhibits in vitro replication (Gruss et al., 1990; Ishimi, 1992; Gruss et al., 1993). EM analysis of the DNA templates in presence of nuclear extract revealed a highly compacted sphere of protein-DNA complexes (Gruss et al., 1990). This could be one of the reasons for the inhibition of the in vitro replication by crude yeast whole-cell extract. However, the fact that we were not able to overcome the inhibition by first
setting up the initiation complex indicates that it was not the compaction of the DNA template alone that led to the abolishment of the replication, because inhibition of in vitro replication by chromatin assembly has been successfully overcome by first setting up the initiation complex or by preincubation of the template DNA with T-antigen (Ishimi, 1992; Gruss et al., 1993). Furthermore, in vitro replication of the same template DNA was inhibited, but not abolished upon the addition of the same amount of yeast proteins (1μg to 10μg) derived from fractions of partially purified yeast.

From these data we conclude that the crude whole-cell yeast extract contains factors that specifically inhibit in vitro replication and that not solely the amount of added proteins is responsible for the abolishment of the in vitro replication reaction.

As a next step, we tried to circumvent this problem by partially purifying the crude whole-cell extract with the aim of eliminating factors that inhibit the in vitro replication. EMSAs represented a convenient and fast method to monitor the fractionation for RFB activity, since we expected the factor(s) in question to be a DNA binding protein(s). However, because protein extracts, even when partially purified, contain a lot of proteins with an affinity to DNA, it proved to be difficult to distinguish between band-shift activities that could be due to a DNA binding protein involved in replication arrest or just unspecific proteins with a high affinity to DNA. Using homologous and heterologous competition experiment, we succeeded in identifying a protein that specifically binds to a sequence essential for the RFB. Furthermore, in vitro replication was not abolished by the addition of up to 10μg of such a yeast fraction containing this specific band-shift activity, suggesting that we successfully eliminated factors that inhibit the in vitro replication reaction. However, no accumulation of RIs was observed when yeast RFB sequences fused to SV40 based plasmids were replicated in vitro in presence of these band-shift fractions. One possible explanation for this finding is that the protein binding to this sequence is not involved in replication arrest. Such a protein could, for instance, be involved in the regulation of transcription or recombination, since regulatory sequences for transcription, as well as sequences involved in mitotic recombination, overlap the sequence required for the RFB (see introduction 3.2). Alternatively, additional factors, which are not present in the partially purified yeast extract, may be
required for the replication arrest. Fob1p, for instance, is a good candidate for such a missing factor (see later).

### 3.5.3 Fob1p and its expression in E. coli

The identification of FOB1, a yeast gene required for the RFB (Kobayashi and Horiuchi, 1996), provided us with a trans-acting factor involved in the replication arrest at the RFB. Inspired by the prokaryotic system, where DNA binding proteins stall oncoming replication forks at specific sites, the Ter-sites (see (Hill, 1992; Baker, 1995; Bussiere and Bastia, 1999) and references therein) we deemed the Fob1p as a candidate for such a DNA-binding protein. Remarkably, only very weak similarities can be found between the Fob1p and other proteins listed in the databases so far. Thus, it was not possible to identify protein domains by searching for similarities to known proteins listed in the databases, which would have allowed us to assign functions, such as DNA-binding, to regions of the Fob1p.

Therefore, we expressed the whole Fob1p in E. coli as a GST fusion protein (Smith and Johnson, 1988). The N-terminal GST fusion tag was primarily chosen because it can promote the solubility of fusion proteins (G. Maga and U. Hübscher, personal communication). Moreover, an N-terminal GST offers additional advantages. Besides its function as purification tag, it can also stabilise the fusion protein. For instance, the Fob1p is predicted to be an unstable protein in E. coli by the N-end rule (Bachmair et al., 1986; Tobias et al., 1991). A GST tag at the N-terminus of the protein might help to stabilise the fusion protein.

The major problem we encountered during the bacterial expression was the poor yield of soluble Fob1p. As a direct consequence of this, the affinity purification over the GST fusion tag was less efficient than it could be, leading to bacterially expressed fusion protein of relatively low purity. Up to 50% of the total isolated proteins consisted of co-purifying proteins from E. coli or degradation products of the fusion proteins. These impurities hampered the EMSA analysis of recombinant Fob1p by the appearance of various retarded bands (see Figure 16C). In contrast, expression of the empty
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pGEX-KG vector yielded large quantities of GST that was highly purified after the one-step affinity purification (more than 95% as judged from SDS-PAGE analysis) indicating that the induction of the bacteria and the purification procedure worked properly.

One reason for the poor yield of soluble, bacterially expressed Fob1p could be its poor solubility. The presence of a hydrophobic stretch of amino acids as well as the observation that soluble Fob1p was only obtained when the bacteria were grown at low temperature suggest that some of the bacterially expressed Fob1p was insoluble (Schein and Noteborn, 1989). However, by comparing E. coli total cell protein from IPTG induced cells with non-induced cells, no protein induction was observed, suggesting that the Fob1p is poorly expressed in E. coli, or degraded very quickly. Since only few degradation products were observed in a Western analysis of bacterially expressed Fob1p (Figure 19B), the latter assumption does not seem to be the case. Furthermore, an approach designed to isolate insoluble fusion proteins (Frangioni and Neel, 1993) did not result in a higher yield of purified, bacterially expressed Fob1p. Taken together, the poor yield of Fob1p expressed in E. coli is presumably the result of weak protein expression. Additionally, a fraction of the expressed protein may be insoluble.

The reason for the poor expression of Fob1p could be its toxicity for E. coli. It seems plausible that a protein involved in the arrest of replication could exert a deleterious effect on bacterial growth when overexpressed. However, since our aim was to express a functionally active protein capable of arresting replication forks, we could not circumvent this problem by expressing only parts of the Fob1p. Likewise, in order to increase the solubility of the expressed protein, it would have been a logical step to eliminate the hydrophobic stretch of amino acids. However, as a result, the functionality of the protein might have been severely affected. Thus, we expressed the entire Fob1p, despite of its poor yield. Furthermore, the soluble Fob1p from E. coli seemed to be, at least partially, active, as the EMSAs, the EM analysis and the in vitro replication analysis showed.
3.5.4 The binding of Foblp to sequences required for the yeast RFB

The binding of Foblp to the yeast sequence essential for the RFB was studied by EMSAs. We found that Foblp retarded the RFB-45 oligonucleotide, weakly but in a sequence-specific manner. The oligonucleotide covers 45 bp of the essential 129 bp and is localised directly next to the HpaI restriction site, to which the nascent strands of the arrested replication fork have been mapped (see chapter 2). Thus, the binding site of the Foblp seems to lie within 50 bp of the stalled replication fork, towards the 3' end of the rRNA transcription unit. Taking the spatial need of the replication machinery into consideration, the location of the Foblp binding makes it an ideal candidate for a DNA binding protein capable of stalling the oncoming replication fork.

We did not succeed to further delineate the sequence requirement for the DNA binding of Foblp by EMSA analysis of two mutated oligonucleotides, where previously published putative consensus sequences were deleted (Ward et al.; Hernandez et al., 1993). A more extensive mutational analysis is required to find a consensus sequence for the DNA binding of Foblp. Interestingly, we found a gel shift using an oligonucleotide that is located towards the HindIII site, just at the border of the position mapped for the nascent strands at the minor stop. This could indicate that, as previously proposed (Brewer et al., 1992), there is more than one Foblp binding site present in the sequence essential for the RFB. In contrast, an oligonucleotide covering the region between the two retarded oligonucleotides was not retarded by the Foblp.

Even though the binding of recombinant Foblp to the RFB sequence proved to be specific, it was rather weak. In the best case, about 10% of the oligonucleotides were retarded by Foblp in EMSA analyses. Glutaraldehyde crosslinked Foblp to the RFB sequence and subsequent EM analysis supported these findings. The reason for this weak binding could be that the bacterially expressed protein was not fully active or possessed somehow different binding properties, for instance, because of missing post-translational modifications in E. coli. However, purified GST-Foblp expressed in yeast, using an analogous yeast system based on the pGEX vector type, did not significantly improve the binding of Foblp to RFB-45, as assayed by EMSAs (data not shown). This indicates that the poor DNA binding efficiency of Foblp is not due to post-translational
modifications absent in *E. coli*. A plausible explanation for the low binding efficiency of Foblp is a possible need of additional protein(s) to efficiently bind the cognate DNA sequence. EMSAs with Foblp and partially purified yeast extracts gave no conclusive data supporting this notion.

3.5.5 Towards the *in vitro* reconstitution of the yeast RFB

Insertion of four consecutive yeast DNA fragments, each spanning the sequence essential for the RFB, into an SV40-based plasmid led to an accumulation of RIs upon replication *in vivo* in the presence of Foblp. This means that we have successfully mimicked the RFB *in vitro* to some extent. Furthermore, it suggests that Foblp, most probably through binding to its cognate yeast sequence, can impede the progressing replication fork, implying that either the Foblp *per se* can mediate the stalling of advancing replication forks or, alternatively, that the cytosolic 293 cell extract, used for the *in vitro* replication, provides factors that are able to complement the missing yeast factors.

The signal of the accumulated RIs is distributed evenly over a relatively broad region of the ascending Y-arc. This suggests that the impediment of the replication occurs in all four introduced RFB sequences, with about the same efficiency. Furthermore, the signal indicates a pausing of the replication fork, rather than a block and is relatively weak. Taking the EMSAs with Foblp into account, it is likely that, despite of the four introduced RFB sequences, only a minority of the DNA molecules is bound by the Foblp, and thus only a minor fraction of the replication forks advancing through the RFB sequences is paused. Consistent with this, in an analogous system, in which the murine RFB was reproduced *in vitro*, only a minor fraction of the replicating molecules was stalled (see for instance Figure 3B in (Gerber et al., 1997)). It is reasonable to assume that the *in vitro* reconstituted RFB is less efficient than the RFB *in vivo* in its natural context. There are several potential reasons for this. It seems that the bacterially expressed Foblp is less efficient than the endogenous Foblp, may be because it lacks a co-factor. Furthermore, the replicative helicase, which is a prime target for the protein
mediating the RFB, is the SV40 T-antigen in the in vitro system, whereas in vivo yeast proteins, possibly MCM proteins (Aparicio et al., 1997; Ishimi, 1997), exert this function. It could well be that a replication fork unwound by T-antigen is much less impeded by a yeast protein such as Foblp. Finally, some factors required for the full RFB activity might be absent in the in vitro system. The bound Foblp could slow down the replication fork transiently, but for the orientation-depandant arrest of the replication fork at the RFB additional factors conferring the specificity might be required. In this scenario, the binding of Foblp would act as an inefficient "roadblock" for the approaching replication forks and not until additional factors contributed to a protein complex, a specific and efficient RFB could be constituted.

In the aforementioned murine system, TTF-1 binding of the Sal-box was reported to be indispensable for the stalling of the replication fork. The TTF-1, however, seems to act in concert with other, yet unknown factors, supporting the notion that a protein complex rather than a single protein constitutes the RFB (Gerber et al., 1997). In contrast, a single protein acting as monomer or homodimer is required for the replication arrest at the Ter-sites in prokaryotes (Baker, 1995; Hill, 1992; Bussiere and Bastia, 1999). The yeast homologue of TTF-1 is the Reb1p (Evers et al., 1995). The Reb1p is a DNA binding protein and, as the TTF-1, it is an essential component for termination of rRNA transcription by polymerase I (Bartsch et al., 1988; Lang and Reeder, 1993). The Reb1p binding site is located in the rDNA enhancer, in vicinity, but outside of the sequence required for the RFB, towards the 3' end of the rRNA transcription unit. Therefore, the Reb1p alone is very unlikely to mediate the RFB. However, this does not exclude that the Reb1p interacts with Foblp in order to reconstitute a fully active RFB. It has been reported that, in addition to the HindIII-HpaI fragment essential for the RFB, sequences located towards the 3' end of the rRNA transcription unit, the HindIII-EcoRI fragment encompassing the Reb1p binding site, were required to fully reconstitute the RFB on a episomal yeast plasmid (Brewer et al., 1992). Moreover, the Reb1p might provide a functional link between replication termination and polymerase I transcription. On the one hand, the RFB was found only in front of transcriptionally active rRNA genes (Lucchini and Sogo, 1994), but on the other hand, an RFB was also found in a RNA polymerase I mutant strain lacking ongoing transcription by polymerase I (Nogi et
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al., 1991; Brewer et al., 1992). These at first glance contradictory data could be reconciled by the finding that this mutant strain contained open, i.e. non-nucleosomal, rDNA enhancers (Dammann et al., 1995). It has been suggested that nucleosome disruption might be required for transcription termination as well as for replication termination. TTF-1 has been reported to determine the chromatin architecture of the active rRNA promoter (Langst et al., 1998). By analogy, the yeast equivalent Reblp may be involved in defining a transcription-competent chromatin architecture. Thus, it is tempting to speculate that a set of interacting regulatory factors, to which Reblp and Foblp belong, co-ordinate the processes of transcription and replication termination, thereby disrupting the nucleosomes.

3.5.6 Future directions

The molecular mechanism of the yeast RFB seems to be more complex than the prokaryotic replication arrest at the Ter-sites, where a single trans-acting factor is sufficient to stall the replication fork. So far, Foblp is the only known protein implicated in the yeast RFB. Thus, it could serve as an anchor point to identify proteins involved in the RFB, as well as processes that are functionally linked to the RFB. Furthermore, the relationship between the RFB, transcription termination and mitotic recombination could be examined. Consequently, the identification of proteins that interact physically with Foblp is of considerable interest. A promising approach to find such interacting proteins is a two-hybrid screen of a S. cerevisiae cDNA library using Foblp as bait (Fields and Song, 1989). Proteins that are found to interact with Foblp in this assay could then be subjected to the assays established during this work. For instance, EMSAs and in vitro replication assays could be supplemented with Foblp and its putatively interacting proteins. Furthermore, the physical interaction could be verified by biochemical means such as GST-pulldown assay or immunoprecipitation with the polyclonal α-Fob1 antibody. As an alternative to the two-hybrid screen of a yeast cDNA library, a selection of candidate proteins, possibly involved in the RFB, such as Reblp for instance, could be directly assayed for interactions with the Foblp in a yeast two-hybrid
system, using Fob1p as bait and the candidate protein as prey or vice versa. Moreover, the candidate proteins can be tested together with Fob1p in the above-mentioned assays.

The availability of the α-Fob1 antibody offers new possibilities. A purification procedure to isolate Fob1p from nuclear yeast extracts could be established, since the fractionation can be monitored for the presence of Fob1p by Western blotting. Alternatively, it might be possible to purify Fob1p efficiently with an affinity column using the α-Fob1 antibody. Such a procedure may also lead to the identification of co-purifying proteins, if they interact tightly with the Fob1p.

An attractive assay to test the chromatin binding of proteins in vivo has recently been described (Hecht et al., 1996; Aparicio et al., 1997). This assay involves formaldehyde crosslinking of living cells, thereby crosslinking the proteins, Fob1p in this case, to the chromatin. The Fob1p could be immunoprecipitated by the polyclonal antibody, the crosslinks subsequently reversed and the associated DNA could be subjected to PCR or Southern analysis. The binding of Fob1p to the RFB region in yeast, even if it was mediated by another protein(s), could be demonstrated by this method.

Taken together, the overexpressed, purified Fob1p together with the polyclonal antibody raised against Fob1p, as well as the assays established in this work provide a variety of possibilities to identify further proteins involved in the RFB. The search for such proteins should prove to be interesting and rewarding.
3.6 Materials and Methods

3.6.1 Strains and culture conditions

Yeast: Yeast strain A1 (see 2.5.1) was used for the preparation of crude whole cell yeast extract. The cells were grown at 30°C in complex medium YPD (1% Bacto yeast extract; 1% Bacto peptone; 2% dextrose) to the indicated OD600.

E. coli: Strains TG1 and BL21 (DE3) were used for the overexpression of Fob1p as a GST fusion protein. The bacteria were grown at 25°C in 2xYTG (16 g/l trypton; 10 g/l yeast extract; 5 g/l NaCl; 5% glucose), 100μg/ml ampicilin. For plasmid isolation strain DH5α was used the cells were grown at 37°C in LB (10 g/l trypton; 5 g/l yeast extract; 5 g/l NaCl) supplied with the appropriate antibiotic.

3.6.2 Oligonucleotides and primers

Only PAGE-purified primers were used for EMSAs. The oligonucleotides were diluted in TE (10mM Tris-HCl, 0.1 mM EDTA, pH 8.0) to the appropriate concentrations (10 to 100 μM). For a summary of all the oligonucleotides used for EMSAs or PCR see appendix 7.2.

3.6.3 Plasmid construction and recombinant DNA technologies

The standard techniques for recombinant DNA technologies were carried out basically as described by Ausubel et al. (Ausubel, 1988), enzyme digestions of DNA according to the manufacturer’s instructions and plasmid isolation with the help of the appropriate Quiagen-Kit, according to the supplied instructions.

Constructs for SV40 in vitro DNA replication: All plasmids constructed for in vitro replication are based on plasmids pSVori and pJYM, respectively (Lusky and Botchan, 1981; Yamaguchi and DePamphilis, 1986).
Three pSVori-derived vectors were constructed by Dr. Mari-Carmen Colomar. They contain either the whole yeast rRNA enhancer defined by an EcoRI-HpaI fragment (Schultz et al., 1993) (termed “enhancer”), the EcoRI-HindIII fragment (termed “terminator” because the site of transcription termination lies within this fragment (Lang and Reeder, 1993)) or the HindIII-HpaI fragment (termed “RFB” because it encompasses the cis-element essential for the RFB (Brewer et al., 1992)) and they were named pSVori-Enh, pSVori-Term, pSVori-RFB, respectively.

pSVori-Enh was constructed the following way: the 2.55 kb EcoRI-EcoRI fragment of the yeast NTS encompassing the enhancer was subcloned into the unique EcoRI restriction site of pSVori. Clones with the HpaI site orientated towards the SV40 ori were chosen, because in these clones the RFB is inserted in a nonpermissive direction for the anticlockwise-moving replication fork. This plasmid (pMCC-1) was digested with HpaI and SphI and the larger of the resulting fragments was ligated to the 632 bp SphI-TaqI fragment of the SV40 genome (the Taq restriction end was made blunt by the Klenow fragment first). The result, pSVori-Enh (pMCC-8), contains the yeast rRNA enhancer fragment in the vicinity (about 650 bp) of the bi-directional SV40 ori. pSVori-RFB (pMCC-9) was constructed by removing the EcoRI-Aval fragment from the enhancer, making the restriction ends blunt and religating the DNA fragment. pSVori-Term (pMCC-10) was constructed as pSVori-RFB, just by cutting with BstBI instead of EcoRI. The size of the constructs is 3377 bp (pSVori-Enh), 3187 bp (pSVori-RFB) and 3256 bp (pSVori-Term), respectively.

pJYM-Enh, pJYM-RFB, pJYM-Term: In order to obtain plasmids of bigger size, three pJYM-based vectors encompassing the above mentioned yeast enhancer, RFB and terminator sequences were constructed as follows: pJYM was digested with BstXI and SfiI. The shorter of the two resulting fragments was replaced by the short EcoRI-SfiI fragment of pSVori-Enh, pSVori-RFB or pSVori-Term, respectively (the staggered BstXI and EcoRI ends had been blunt ended). The resulting plasmids have a size of 8575 (pJYM-Enh), 8385 (pJYM-RFB) and 8454 (pJYM-Term), respectively.

pJYM-RFB2, pJYM-RFB3, pJYM-RFB4: A set of plasmids containing two, three or four RFB sequences in tandem (all of them in a head-to-tail orientation) was constructed. They are based on the pJYM-RFB plasmid and were termed pJYM-RFB2, -3
and 4, respectively. pJYM-RFB2 was constructed as follows: pJYM-RFB was cut with Bsal, made blunt with the Klenow polymerase and ligated with the HindIII-HpaI fragment, which likewise had been made blunt. The plasmid, which has the RFB fragment inserted in the same direction, was chosen and termed pJYM-RFB2. pJYM-RFB3 is based on pJYM-RFB2 and was constructed the same way as described for pJYM-RFB2, just instead of the Bsal restriction site the AvaI site was used. pJYM-RFB4 was constructed applying the same procedure on pJYM-RFB3.

pGEX-Fobl: This construct, which was used for the overexpression of Fobl as a GST-fusion protein in E. coli, is based on the pGEX-KG vector (Guan and Dixon, 1991). The cDNA of the FOBL gene was cloned by PCR (no introns) using Pwo, a high fidelity thermostable DNA polymerase. The forward primer, Fobl-5B, carries a 5' overhang with a BamH1 restriction site, the reverse primer, Fobl-3X, a 5' overhang with an XhoI restriction site. The PCR reaction was carried out using 1 ng of purified genomic DNA (strain A1) as template and included 20 cycles of repeated denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension for 2 min at 72°C. The resulting PCR fragment was purified over an agarose gel, digested with BamH1 and XhoI and ligated to an identically digested pGEX-KG vector. The sequence across the junction of the GST and the introduced FOBL cDNA was determined by the dideoxy chain termination method (Sanger et al., 1977) using the Sequenase 2.0 sequencing kit (Amersham) with primer Fobl-Seq 1.

### 3.6.4 In Vitro DNA Replication

The human cell line 293 was cultured in Dulbecco's modified Eagle's medium (DMEM) and 10% fetal calf serum (FCS). Replication competent, cytosolic 293 cell extracts were prepared exactly as described (Stillman and Gluzman, 1985). Briefly: 20 plates (140 mm) of 70%-80% confluent 293 cells were harvested by scraping the cells off the plates with a rubber policeman. The cells were transferred into a 15ml Falcon tube, washed twice with cold PBS (140mM NaCl, 2.7mM KCl, 10mM Na2HPO4, 1.8mM KH2PO4, pH 7.3) and hypotonic buffer I (20 mM HEPES-KOH (pH 7.5); 5 mM
KCl; 1.5 mM MgCl₂, 250 mM sucrose), resuspended in 5 ml hypotonic buffer II (20 mM HEPES-KOH (pH 7.5); 5 mM KCl; 1.5 mM MgCl₂) and disrupted with six strokes in a 5 ml glass Dounce homogeniser. The mixture was kept on ice for 15 min, subsequently centrifuged for 10 min at 10000 rpm (Sorvall, Hb-4) and the resulting supernatant was adjusted to 100 mM NaCl. As a final step ultracentrifugation was carried out at 31000 rpm (SW60, Beckman) for one hour and the crude cytosolic extract was collected by tube puncture.

Standard in vitro replication was carried out in 50 µl reaction volume with 100 ng to 200 ng template DNA in 30 mM HEPES-KOH (pH 7.8); 0.5 mM DTT; 3 mM MgCl₂; 2 mM ATP; 80 µM (each) CTP, GTP, and UTP; 0.1 mM (each) dATP, dGTP and dTTP; 5 mCi each of [α-³²P]dCTP (3,000 Ci/mmol); 40 mM creatinphosphate; 1.2 µg/µl of creatine kinase; 230 µg of S100 cytoplasmic protein extracted from human 293 cells; and 1 µg T antigen. Replication was allowed proceeding for 15 min to 45 min at 37°C, and the reaction was stopped by the addition of 0.7% SDS, 20 mM EDTA. After digestion with proteinase K (0.2 mg/ml) at 37°C for 2 hours, the DNA was purified by phenol extraction, gel filtration over a Sephadex G50 column (Roche Molecular Biochemicals), and ethanol precipitation. Resuspended DNA was digested with KpnI and ClaI and subjected to 2D gel electrophoresis. The gel was dried and the signals were detected by autoradiography.

2D gel analysis was carried out as described previously (Brewer and Fangman, 1987) with the following parameters. First dimension: 0.4% agarose (Sigma), 0.5x TBE (45mM Tris, 45mM boric acid, 2mM EDTA, pH 8.0), 1V/cm for 16 hours at room temperature. Second dimension: 1% agarose, 0.5xTBE, 0.3 µg/µl Ethidium Bromide, 4V/cm for 8 hours at 4°C.

### 3.6.5 Yeast whole-cell extracts

Transcription competent yeast whole cell extracts were prepared essentially as described by Schultz et al. (Schultz et al., 1991). Yeast cells were harvested at an OD₆₀₀ of about 1-2 by pouring the culture through crushed ice into 500 ml centrifuge bottles. The cells were centrifuged at 4000 rpm in a GSA rotor, washed twice with cold bides-
tilled H₂O, once with YEB solution (100mM HEPES-KOH, 245mM KCl, 1mM EDTA, 5mM EGTA-KOH) and subsequently resuspended in 1.3 vol. (wet weight of cells) YEB. This cell paste was transferred to a mortar filled with liquid nitrogen. The frozen cells were broken by extensively grinding them in small batches leading to a frozen powder that was thawed at 4°C by addition of 2 vol. of YEP containing protease inhibitors (0.2mM PMSF, 10mM TPCK, 25µg/ml Benzamidine-HCl, 5µg/ml Leupeptin, 3.5µg/ml Pepstatin, 10µg/ml Aprotinin). The suspension is immediately transferred to a centrifuge tube and spun for 2 h at 100,000g in an ultracentrifuge. The resulting supernatant is collected by tube puncture and dialysed against 50 vol. of YDB (20mM HEPES-KOH, 50mM KCl, 0.05mM EDTA, 5mM EGTA-KOH, 2.5mM DTT, 20% Glycerol, 0.2mM PMSF, 0.5 µg/ml Leupeptin).

Protein concentrations were determined according to the Bradford method (Bradford, 1976) with the Bio-Rad protein assay reagent using bovine serum albumin as a standard. Typically, a protein concentration of 15 to 20 µg/ml was obtained.

3.6.6 Fractionation of crude yeast whole-cell extract

The fractionation procedure is shown schematically in Figure 12. In a first step about 50 mg of crude whole-cell extract was loaded on a Q-Sepharose column equilibrated in Buffer I (50mM Tris-HCl pH 7.5, 20% glycerol, 1mM ATP, 1mM DTT, 1mM EDTA and 2mM MgCl₂). The column was washed with 4 volumes of buffer I and then eluted with a linear gradient from 0 to 1M NaCl. Protein elution was monitored by absorption at 260nm and all fractions containing eluted proteins were pooled and loaded onto a Mono Q FPLC column, equilibrated in buffer I. After washing the column with 4 volumes of buffer I and protein elution by a linear gradient of 0 to 1M NaCl, the collected fractions were monitored for binding to the RFB-45 oligonucleotide EMSAs. Fractions retarding the oligonucleotide sequence-specifically were pooled, diluted with Buffer II (10mM KPO₄, pH 7.2, 20% glycerol, 50mM KCl) and loaded onto a Heparin column equilibrated in Buffer II. The column was washed with 4 volumes of buffer II
and subsequently eluted with a linear gradient from 0.05mM to 0.7 mM KCl. The flow-through was reloaded onto a Mono Q column equilibrated in buffer I and eluted with a linear gradient of 0 to 1M NaCl.

### 3.6.7 Electromobility shift assays

2 pmol of single-stranded, synthetic oligonucleotide were end-labelled with \([\gamma^{32}\mathrm{P}]\mathrm{ATP}\) (3000 Ci/mmol) by polynucleotide kinase (New England Biolabs) according to the manufacturer's instructions. The unincorporated \([\gamma^{32}\mathrm{P}]\mathrm{ATP}\) was removed using a Sephadex G50 column. 4 pmol unlabelled complementary oligonucleotide of identical length were added. MgCl₂ was adjusted to a final concentration of 5mM and the mixture was heated for 5' to 100°C and subsequently left at room temperature for several hours.

A typical DNA binding reactions was carried out in 20µl "binding buffer" (10 mM Tris pH 7.8, 50mM KCl, 50mM NaCl, 1mM MgCl₂, 1mM EDTA, 5% glycerol) using 0.005 pmol of labelled, synthetic oligonucleotide, 0.5 to 10 µg of poly[dA-dT] and either yeast extract or bacterially expressed Fob1p. Additionally, 1mM ATP was added to reactions with yeast extract. The protein amounts present in the mixtures varied from 5 to 20µg in the case of yeast extract and from 50 to 500 ng for purified, bacterially expressed Fob1p. The binding reaction was carried out for 30 min at 4°C. Protein-DNA complexes were separated from free oligonucleotides at 0.15 V/cm in a 4% non-denaturing polyacrylamide gel in 0.5 x TBE (45mM Tris, 45mM boric acid, 2mM EDTA, pH 8.0), that was prerun for 1 h at 0.15 V/cm. The gels were dried and the signals detected by autoradiography.

### 3.6.8 EM analysis

250 ng of pSVori-Enh plasmid, linearised with \(NcoI\), was incubated with approximately 200 ng of purified, recombinant Fob1p. The binding reaction was carried out exactly as for the EMSAs (at 4°C, 30 min) in the same binding buffer as used for the EMSAs, just with TAE instead of Tris (10mM, pH 7.8). The samples were fixed in 0.1% of Glutaraldehyde-
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hyde for 30 min at room temperature and subsequently purified over a Sepharose 4B column. The DNA was spread by the mica adsorption method exactly as described (Sogo et al., 1987). A droplet of DNA sample at a concentration of ~0.2 μg/ml containing 5 mM Mg-acetate was adsorbed on a sheet of mica. After washing for 3 hours in bidistilled water the mica containing the DNA samples was rotary shadowed with 750 Hz platinum at an angle of ~1.5° and coated with carbon for preparing replicas. DNA contour length measurements were made with a Hewlett-Packard digitizer on photographic prints.

3.6.9 Overexpression of Fob1p in E. coli

100ml 2xTYG/100μg/ml ampicillin were inoculated with a single colony of E. coli cells and incubated for about 16 hours at 37°C. 1ml to 5ml of the culture was diluted 1:200 into fresh 2xTYG/100μg/ml ampicillin and grown, vigorously shaking, until the OD₆₀₀ reached 0.5. Protein expression was induced by adding IPTG to a final concentration of 1mM and the incubation was continued for 4 to 6 hours. The cells were sedimented in a GS3 rotor at 5000 rpm for 10 min and the pellet was resuspended in 25μl/ml cell culture of ice-cold PBS (140mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄, 1.8mM KH₂PO₄, pH 7.3), 1mM DTT, 1mM PMSF. Subsequently, the cells were disrupted by sonication in short bursts and after adding 20% Triton-X 100 to a final concentration of 1%, the mixture was gently swirled and incubated on ice for 30min. The cell debris were pelleted by centrifugation at 15000 rpm in a SS34 rotor for 20min and 20μl/100ml cell culture of Glutathione-Sepharose 4B (Pharmacia), pre-equilibrated in PBS, was added. The mixture was incubated for 1 hour at room temperature, keeping the Sepharose in solution by wheeling. Subsequently, the slurry was centrifuged at 1500 rpm for 5 min in a CR-422 tabletop centrifuge and the beads were washed 3x with 10 Glutathione-Sepharose volumes. The GST-Fob1p was eluted by addition of 1 Glutathione-Sepharose volume of 10mM reduced glutathione in 50mM Tris-HCl, pH 8.0. The Fob1p was eluted by cleavage with 0.25 cleavage Units of Thrombin, wheeling overnight at 4°C.

The yield and purity of the eluted proteins were assessed by SDS-PAGE (Laemmli, 1970) stained with Coomassie Blue or by silver staining (Heukeshoven and
Dernick, 1988). A protein molecular weight standard (broad range standard of Biorad) was run in parallel. The yield of a 500ml culture was about 100 to 200μg of Fob1p in the best case.

3.6.10 Production of a polyclonal α-Fob1 antiserum in rabbits

*Preparation of highly purified Fob1 antigen:* Bacterially expressed, purified Fob1p (lacking the GST-tag) was re-purified over a preparative SDS-PAGE by cutting out a gel slice at the position where the Fob1p migrated (the gel was not stained before). The acrylamide containing the Fob1p was crushed by nebulization (achieved by centrifugation through a tiny hole).

*Immunisation procedure:* Pre-immune serum of six rabbits was tested in a Western blot of crude whole-cell yeast extract. The two rabbits (termed #5 and #6) with the pre-immune serum that give rise to smallest cross-hybridisation signal were chosen for immunisation. For immunisation, 1ml of the nebulized Fob1p-acrylamide, with an estimated amount of 200μg pure Fob1p, in PBS (140mM NaCl, 2.7mM KCl, 10mM Na2HPO4, 1.8mM KH2PO4, pH 7.3) was emulsified with 1ml of complete Freund’s adjuvant. The rabbit was prime immunised by intradermal injection of the emulsion. The first booster immunisation was administered 19 days later, the second after another 12 days using equal amounts of identically prepared antigen in a 1:1 emulsion with incomplete Freund’s adjuvant.

*Preparation of antisera:* The rabbits were bled from the ear artery, 10 days after each booster immunisation. The collected blood (50ml to 100ml) was allowed to stand for 4 hours at room temperature and left at 4°C overnight. The resulting blood clot was removed, the serum transferred into a centrifugation tube and any remaining red blood cells and cell debris pelleted by centrifugation at 5000g for 10 min. Finally, the antisera were tested on a Western blot by detection of Fob1p with a dilution series of the antiserum.
3.6.11 Western blot analysis

Bacterially expressed Fob1p, crude yeast extracts or partially purified yeast extracts were separated on a 10 % SDS-PAGE (Laemmli, 1970). A protein molecular weight standard (broad range standard of Biorad) was included. After the run, a nitrocellulose membrane (Protan nitrocellulose BA85, 0.45 μm, Schleicher and Schuell) was placed onto the separating gel and they were sandwiched with 9 3MM papers of the appropriate size, pre-equilibrated in “semi-dry blotting buffer” (2.93 g/l glycine, 5.81 g/l Tris 0.75 g/l SDS, 20% v/v methanol). The proteins were transferred in a semi-dry electrophoretic transfer cell (Bio-Rad) applying 1mA/cm² for 45 min. The membrane was stained with Ponceau S (Bio-Rad) to visualise the transfer and position of the protein standards. Subsequently, the staining was reversed by soaking the membrane 10 min in water. The membrane was probed with pre-immune serum or with the polyclonal α-Fob1p antiserum at a 1:2000 dilution. As secondary antibody we used a Horseradish peroxidase-coupled goat anti-rabbit antibody (Pierce) diluted 1:3000. All dilutions were made in PBS, 5 % (w/v) non-fat dry milk, 0.3 % (v/v) Tween-20. Bands were visualised by chemiluminescence.

3.6.12 Immunoprecipitation of Fob1p

10 μg of crude whole-cell yeast extract or partially purified extract was incubated with 20 μl of pre-immune serum or 20 μl α-Fob1p antiserum overnight at 4 °C. 0.02 g of protein A-Sepharose (Pharmacia), pre-swollen in 20mM Tris-HCl pH 7.5 was subsequently added and the incubation continued for additional 4 h at 4 °C. The reaction mixture was then centrifuged for 10 min at 12000g, the supernatant was removed and the pellet was washed 3 times with PBS (140mM NaCl, 2.7mM KCl, 10mM Na2HPO4, 1.8mM KH2PO4, pH 7.3). After the last centrifugation, 4x Laemmli buffer (0.25M Tris, 8% SDS, 10% glycerol, 10% 2-mercaptoethanol, 0.1% bromphenol blue) was added, the mixture was boiled and shortly centrifuged as above. The pellet was directly subjected to SDS-PAGE and Western analysis as described above.
4 LITERATURE


5 APPENDIX

5.1 Abbreviations

\( \mu g \)  micrograms
\( \mu l \)  microlitres
°C  Celsius degree
APS  ammonium persulfate
ARS  Autonomous replication sequence
ATP  adenosine triphosphate
BSA  bovine serum albumin
Ci  Curie
cm  centimetre
cpm  counts per minute
CsCl  caesium chloride
CTP  cytosine triphosphate
ddNTP  dideoxy nucleotide triphosphate
DE81  DEAE cellulose paper
DNA  deoxyribonucleic acid
dNTP  deoxy nucotide triphosphate
ds  double-stranded
DTT  Dithiothreitol
EDTA  ethylenediamin-tetraacetat-di-sodium-salt
EGTA  \([\text{Ethylenebis(oxyethylenenitrilo})]\text{tetraacetic acid}\)
EM  electron microscopy
EMSA  electromobility shift assay
endo VII  T4 endonuclease VII
EtBr  Ethidium bromide
EtOH  ethanol
Fob1p  fork blocking protein 1
\( g \)  grams
GST  glutathione-S-transferase
GTP  guanosine triphosphate
h  hour(s)
kb  kilo bases
KCl potassium chloride
kD  kilo Dalton
M  molar
MCM minichromosome maintenance
mg  milligrams
MgCl₂ magnesium chloride
min minute(s)
ml  millilitres
mM millimolar
NaAc sodium acetate
NaCl sodium chloride
ng  nannogram
nm  nannometers
nt  nucleotide(s)
NTS non-transcribed spacer
PAGE polyacrylamide gel electrophoresis
pmol picomol
PMSF phenylmethane sulfonyl fluoride
rDNA ribosomal deoxyribonucleic acid
RFB replication fork barrier
RNA ribonucleic acid
rpm revolutions per minute
rRNA ribosomal ribonucleic acid
RT room temperature
SDS sodium dodecyl sulfate
sec second(s)
ss  single-stranded
TE 10 mM Tris-HCl pH 8, 0.1 mM EDTA pH 8.0
Tm  melting temperature
Tris HCl Tris-(hydroxymethyl)-amino methane hydrochloric acid
TTP thymidine triphosphate
U  Unit(s)
v/v  volume/volume
vol  volume
w/v  weight/volume
5.2 Compilation of Oligonucleotides and Primers

*Oligonucleotides used for EMSAs:* The putative consensus sequences for a DNA binding protein proposed by Hernandez et al. (Hernandez et al., 1993, single line) and by Brewer et al. (personal communication, double line), respectively, are underlined.

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*Primers annealing to yeast rDNA used for Primer Extension and PCR:*

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## 5 Appendix

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**Purposes:**
1. High resolution mapping of the nascent lagging strand by primer extension (primer anneals to the replicated part of the replication fork stalled at the RFB).
2. High resolution mapping of the parental lagging strand by primer extension (primer anneals to the unreplicated part of the replication fork stalled at the RFB).
3. Generation of double-stranded probes encompassing the RFB for Southern analysis.
4. Generation of strand specific probes by primer extension.
5. Amplification of Fobl cDNA for subcloning into the expression vector pGEX-KG. Both primers contain a 10 bp 5' overhang accommodating a BamHI (primerFobl-5B) and an XhoI (primerFobl-3X).
5.3 Maps of Constructs

Colour code:
- Black: pBR322
- Blue: SV40
- Red: yeast

1. EcoRI digest
2. Ligation

SphI-TaqI fragment (437bp, TaqI was blunted) from SV40 genome

1. cut with HpaI-SphI
2. take long 2372bp fragment
3. Ligate with 437 bp fragment

1. cut with AvaI-EcoRI
2. make blunt
3. relegate 3183bp fragment

1. cut with AvaI-BstBI
2. make blunt
3. relegate 3253bp fragment

143
1. cut pSVori with SfiI-EcoRI, make EcoRI blunt
2. cut pJYM with SfiI-BstBI, make BstXI blunt
3. ligate small fragments encompassing RFB, enhancer, terminator, respectively to 7758 fragment of pJYM
1. cut pJYM-RFB with BstBI, make blunt
2. purify HindIII-HpaI fragment, make HindIII blunt
3. ligate the two fragments, select for clone in which both RFB sequences orientated in the same way (pJYM-RFB2)
4. cut pJYM-RFB2 with AvaI, insert above mentioned Hind-HpaI fragment (blunt) in the same orientation (pJYM-RFB3)
5. same procedure as in 4. using pJYM-RFB3 (pJYM-RFB4)
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Curriculum Vitae

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Mai 1997
I. Departement-Biologie-Symposium of the ETH Zürich, Davos: Poster presentation.

March 1997
USGEB-Congress in Genf: Poster presentation.

October 1996
Swiss Yeast Meeting in Zürich: Poster presentation.

September 1996
EMBO-Workshop: Molecular Biology of DNA Replication in Weggis: Poster presentation.

March 1995
USGEB-Congress in Fribourg: Poster presentation. Winner of prize for best Poster.

Publications
